Biomarkers in Disease:
Methods, Discoveries and Applications
Series Editor: Victor R. Preedy

SPRINGER
REFERENCE

Vinood B. Patel · Victor R. Preedy *Editors*

Biomarkers in Liver Disease

Springer

Biomarkers in Disease: Methods, Discoveries and Applications

Series Editor
Victor R. Preedy
Diabetes and Nutritional Sciences Research Division
Faculty of Life Science and Medicine
King's College London
London, UK

In the past decade there has been a major sea change in the way disease is diagnosed and investigated due to the advent of high-throughput technologies, such as microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases etc. In many instances these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics and bioinformatics these markers have been used to identify individuals with active disease or pathology as well as those who are refractory or have distinguishing pathologies. Unfortunately techniques and methods have not been readily transferable to other disease states and sometimes diagnosis still relies on single analytes rather than a cohort of markers. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the formulation of Biomarkers in Disease.The series covers a wide number of areas including for example, nutrition, cancer, endocrinology, cardiology, addictions, immunology, birth defects, genetics and so on. The chapters are written by national or international experts and specialists.

Series Titles

1. General Methods in Biomarker Research and Their Applications
2. Biomarkers in Cancer
3. Biomarkers in Cardiovascular Disease
4. Biomarkers in Kidney Disease
5. Biomarkers in Bone Disease
6. Biomarkers in Liver Disease

More information about this series at http://www.springer.com/series/13842

Vinood B. Patel • Victor R. Preedy
Editors

Biomarkers in Liver Disease

With 162 Figures and 124 Tables

Springer

Editors
Vinood B. Patel
Department of Biomedical Science
Faculty of Science and Technology
University of Westminster
London, UK

Victor R. Preedy
Diabetes and Nutritional Sciences Research Division
Faculty of Life Science and Medicine
King's College London
London, UK

ISBN 978-94-007-7674-6 ISBN 978-94-007-7675-3 (eBook)
ISBN 978-94-007-7676-0 (print and electronic bundle)
DOI 10.1007/978-94-007-7675-3

Library of Congress Control Number: 2017933321

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer Science+Business Media B.V.
The registered company address is: Van Godewijckstraat 30, 3311 GX Dordrecht, The Netherlands

Preface

In the present volume, *Biomarkers in Liver Disease*, we have sections on:

1. ***General Aspects and Introductory Material***
2. ***Body Fluids, Tissue, and Specific Biomarkers***
3. ***Genetic, Histological, Physical, and Imaging Methods***
4. ***Specific Diseases and Conditions***
5. ***Resources***

The editors recognize the difficulties in assigning particular chapters to particular sections, as some chapters can fit into more than one section. Nevertheless, the book has enormously wide coverage. Platforms and techniques include, for example, immunological, biochemical, histochemical methods, bioelectrical impedance analysis, and others. Conditions and biomedical areas encompass: adiposity, alcohol misuse, ascites, cirrhosis, diabetes, end-stage liver disease, extracellular matrix remodeling, hepatic fibrosis, hepatitis C virus infection, hepatocellular carcinoma, liver diseases in general, liver transplantation, nonalcoholic fatty liver disease, nutritional interventions, paracetamol-induced acute liver failure, portal hypertension, and tumor staging. Analytes and measures include alanine aminotransferase, alpha-fetoprotein, antioxidant response, AST-to-platelet ratio index, bilirubin, body fluids, CD133, CD163, cell-free DNA, cortisol, cytokines, EpCAM, extracellular vesicles, fibrinogen, hydroxyproline, inflammatory biomarkers, microRNAs, monocyte chemotactic protein-1, osteopontin, phosphatidylethanol, polymorphisms, PTX3, SCCA-IgM, scoring systems, sialic acids, sialidases, sialyltransferases, traditional markers, tumor staging, type VI collagen, VCAM-1, and YKL-40. There are also many other analytes and conditions described within this volume.

Finally, the last chapter is devoted to locating resource material for biomarker discovery and applications.

The chapters are written by national or international experts. This book is designed for clinical biochemists, hepatologists, gastroenterologists with sub-area interests, specialists working within the field of organ disease and treatments, health

scientists, epidemiologists, doctors and nurses, from students to practioners at the higher level. It is also designed to be suitable for lecturers and teachers in health care and libraries as a reference guide.

The Editors

Series Preface

In the past decade, there has been major changes in the way diseases are diagnosed and investigated due to the advent of high-throughput technologies and advances in chemistry and physics. This has lead to the development of microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics, and other new platforms. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, and many other conditions too numerous to list here. In many instances, these developments have gone hand in hand with analysis of biomarkers elucidated via traditional methods, such as histopathology, immunoassays, and clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics, and bioinformatics these markers have been used to identify individuals with active disease as well as those who are refractory or have distinguishing pathologies.

Unfortunately, techniques and methods have not been readily transferable to other disease states, and sometimes diagnosis still relies on a single analyte rather than a cohort of markers. Furthermore, the discovery of many new markers has not been put into clinical practice partly because of their cost and partly because some scientists are unaware of their existence or the evidence is at the preclinical stage. There is thus a demand for a comprehensive and focused evidenced-based text that addresses these issues. Hence, the book series **Biomarkers in Disease: Methods, Discoveries and Applications**. It imparts holistic information on the scientific basis of health and biomarkers and covers the latest knowledge, trends, and treatments. It links conventional approaches with new platforms. The ability to transcend the intellectual divide is aided by the fact that each chapter has:

- ***Key Facts (areas of focus explained for the lay person)***
- ***Definitions of Words and Terms***
- ***Potential Applications to Prognosis, Other Diseases, or Conditions***
- ***Summary Points***

The material in ***Potential Applications to Prognosis, Other Diseases, or Conditions*** pertains to speculative or proposed areas of research, cross-transference to

other diseases or stages of the disease, translational issues, and other areas of wide applicability.

The series is expected to prove useful for clinicians, scientists, epidemiologists, doctors and nurses, and also academicians and students at an advanced level.

The Editors

Contents

About the Editors

Vinood B. Patel
Department of Biomedical Science
Faculty of Science and Technology
University of Westminster
London, UK

Dr. Vinood B. Patel is currently a Reader in Clinical Biochemistry at the University of Westminster and honorary fellow at King's College London. He presently directs studies on metabolic pathways involved in tissue pathology particularly related to mitochondrial energy regulation and cell death. Research is being undertaken to study the role of nutrients, antioxidants, phytochemicals, iron, alcohol, and fatty acids in tissue pathology. Other areas of interest include identifying new biomarkers that can be used for diagnosis and prognosis of liver disease, understanding mitochondrial oxidative stress in Alzheimers disease, and gastrointestinal dysfunction in autism. Dr. Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his Ph.D. in Protein Metabolism from King's College London in 1997. His postdoctoral work was carried out at Wake Forest University Baptist Medical School studying structural-functional alterations to mitochondrial ribosomes, where he developed novel techniques to characterize their biophysical properties. Dr. Patel is a nationally and internationally recognized liver researcher and was involved in several NIH-funded biomedical grants related to alcoholic liver disease. Dr. Patel has edited biomedical books in the area of nutrition and health, autism, and biomarkers and has published over 150 articles, and in 2014 he was elected as a Fellow to The Royal Society of Chemistry.

Victor R. Preedy is a senior member of King's College London. He is also Director of the Genomics Centre and a member of the Faculty of Life Sciences and Medicine.

Professor Preedy graduated in 1974 with an Honours Degree in Biology and Physiology with Pharmacology. He gained his University of London Ph.D. in 1981. In 1992, he received his Membership of the Royal College of Pathologists, and in 1993 he gained his second doctoral degree for his outstanding contribution to protein metabolism in health and disease. Professor Preedy was elected as a Fellow to the Institute of Biology in 1995 and to the Royal College of Pathologists in 2000. Since then he has been elected as a Fellow to the Royal Society for the Promotion of Health (2004) and The Royal Institute of Public Health (2004). In 2009, Professor Preedy became a Fellow of the Royal Society for Public Health, and in 2012 a Fellow of the Royal Society of Chemistry. In his career, Professor Preedy has carried out research at the National Heart Hospital (part of Imperial College London), The School of Pharmacy (now part of University College London), and the MRC Centre at Northwick Park Hospital. He has collaborated with research groups in Finland, Japan, Australia, USA, and Germany. He is a leading expert on the science of health and has a long standing interest in biomarkers for over 30 years especially related to tissue pathology. He has lectured nationally and internationally. To his credit, Professor Preedy has published over 600 articles, which include peer-reviewed manuscripts based on original research, abstracts and symposium presentations, reviews, and numerous books and volumes.

Editorial Advisor

Rajkumar Rajendram
Consultant in Internal and Perioperative Medicine
King Abdulaziz Medical City
Ministry of National Guard Health Affairs
Riyadh, Saudi Arabia

Contributors

Leon A. Adams School of Medicine and Pharmacology, University of Western Australia, Perth, WA, Australia

Nancy E. Aguilar-Olivos Liver Research Unit, Medica Sur Clinic & Foundation, Mexico City, Mexico

Jawad Ahmad Division of Liver Diseases and Recanati-Miller Transplantation Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Krasimira Aleksandrova Nutrition, Immunity and Metabolism Start-up Lab, Department of Epidemiology, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany

Ahmad H. Alghadir Department of Rehabilitation Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Riyadh, Saudi Arabia

A. Ata Alturfan Department of Sciences, Institute of Forensic Sciences, Istanbul University, Istanbul, Turkey

Agnieszka Bakula Department of Gastroenterology, Hepatology, Nutrition Disorders and Paediatrics, The Children's Memorial Health Institute, Warsaw, Poland

Pedro Baptista Digestive Pathology Group, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain

Arpita Basu Department of Nutritional Sciences, 301 Human Sciences, Oklahoma State University, Stillwater, OK, USA

Paramita Basu Department of Biology, Texas Woman's University, Denton, TX, USA

Julia Beck Chronix Biomedical GmbH, Göttingen, Germany

A. Biasiolo Department of Medicine, University of Padua, Padua, Italy

Anna Blum Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany

Laura Boemeke Post Graduate Program in Medicine: Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil

Radan Bruha General University Hospital, Charles University in Prague, Prague 2, Czech Republic

Anthony W. H. Chan Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Shatin, NT, Hong Kong

Stephen L. Chan State Key Laboratory of Oncology in South China, Department of Clinical Oncology, Chinese University of Hong Kong, Shatin, NT, Hong Kong

Eun-Ju Chang Department of Biomedical Sciences, University of Ulsan College of Medicine, Asan Medical Center, Songpa-gu, Seoul, Republic of Korea

Bongkun Choi Department of Biomedical Sciences, University of Ulsan College of Medicine, Asan Medical Center, Songpa-gu, Seoul, Republic of Korea

Lech Chrostek Department of Biochemical Diagnostics, Medical University of Bialystok, Bialystok, Poland

Maxey Ching Ming Chung Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Miriam Cortes Liver Transplant Unit, Institute of Liver Studies, King's College Hospital, London, UK

Michael H. Court Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA, USA

Karina Ana da Silva Department of Physiological Sciences, Federal University of São Carlos (UFSCAR), São Carlos, São Paulo, Brazil

Maciej Dadalski Department of Gastroenterology, Hepatology, Nutrition Disorders and Paediatrics, The Children's Memorial Health Institute, Warsaw, Poland

Cynthia Aparecida de Castro Department of Physiological Sciences, Federal University of São Carlos (UFSCAR), São Carlos, São Paulo, Brazil

Jeroen de Jonge Department of Surgery, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands

Department of Surgery, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands

Letícia De Nadai Marcon Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Luis Fernando de Sousa Moraes Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Luis del Pozo Yauner Laboratorio de Bioquímica y Estructura de Proteínas, Instituto Nacional de Medicina Genómica, Mexico City, Mexico

Giuseppe Derosa Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for the Study of Endocrine-Metabolic Pathophysiology and Clinical Research, University of Pavia, Pavia, Italy

Laboratory of Molecular Medicine, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

Deepika Devuni Division of Liver Diseases and Recanati-Miller Transplantation Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Sandra Aparecida dos Reis Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Saad Elias Department of Internal Medicine A, Galilee Medical Center, Nahariya, Israel

Bar Ilan University, Safed, Israel

Walid Elmoghazy Hepatobiliary and Liver Transplant Surgery, Hamad Medical Corporation (HMC), Doha, Qatar

Ebru Emekli-Alturfan Department of Biochemistry, Faculty of Dentistry, Marmara University, Istanbul, Turkey

Guido Engelmann Department of Pediatrics, Lukas Hospital, Neuss, Germany

G. Fassina Xeptagen SpA, VEGA Park, Venice, Italy

Giuseppe Fede Department of Clinical and Experimental Medicine, University of Catania- Garibaldi Hospital, Catania, Italy

Sabrina Alves Fernandes Post Graduate Program in Bioscience and Rehabilitation; Post Graduate Program in Rehabilitation and Inclusion, IPA Methodist University, Porto Alegre, RS, Brazil

Sven Francque Department of Gastroenterology and Hepatology, Antwerp University Hospital, Edegem, Belgium

Laboratory of Experimental Medicine and Pediatrics, University of Antwerp, Wilrijk, Belgium

Sami A. Gabr Department of Rehabilitation Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Riyadh, Saudi Arabia

Department of Anatomy, Faculty of Medicine, Mansoura University, Mansoura, Egypt

A. Gallotta Xeptagen SpA, VEGA Park, Venice, Italy

Juan Carlos García-Cañaveras Unidad de Hepatología Experimental, Unidad Analítica, Instituto de Investigación Sanitaria, Fundación Hospital La Fe, Valencia, Spain

Ayman A. Ghfar Department of Chemistry, College of Science, King Saud University, Riyadh, Saudi Arabia

Maria do Carmo Gouveia Peluzio Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Henning Grønbæk Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark

Ewa Gruszewska Department of Biochemical Diagnostics, Medical University of Bialystok, Bialystok, Poland

Alicja E. Grzegorzewska Department of Nephrology, Transplantology and Internal Diseases, Poznan University of Medical Sciences, Poznań, Poland

Yi Huang School of Medicine and Pharmacology, University of Western Australia, Perth, WA, Australia

Hartmut Jaeschke Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA

Gary P. Jeffrey School of Medicine and Pharmacology, University of Western Australia, Perth, WA, Australia

Wladimiro Jimenez Biochemistry and Molecular Genetics Service, Biochemistry and Molecular Genetics Department, Hospital Clinic of Barcelona, Barcelona, Spain

Department of Biomedicine, University of Barcelona, Barcelona, Spain

Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Barcelona, Spain

Ewelina Kałużna Department of Molecular Pathology, Institute of Human Genetics, Polish Academy of Sciences, Poznań, Poland

Philipp Kanzow Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany

Department of Preventive Dentistry, Periodontology and Cariology, University Medical Center Göttingen, Göttingen, Germany

Morten A. Karsdal Nordic Bioscience, Biomarkers and Research, Herlev, Denmark

Samy Kashkoush Department of Hepatobiliary Surgery and Liver Transplantation, National Liver Institute, Minufiya University, Minufiya, Egypt

Organ Transplant Center, King Abdullah Specialist Children Hospital (KASCH) National Guard, Riyadh, Saudi Arabia

Konstantin Kazankov Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark

Norihiro Kokudo Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Olena Kolesnikova Department of Hepatology, Government Institution L.T. Malaya Therapy, National Institute of NAMS of Ukraine, Kharkiv, Ukraine

Otto Kollmar Helios Dr. Horst Schmidt Kliniken Wiesbaden, Wiesbaden, Germany

Aleksander Krag Department of Gastroenterology and Hepatology, University of Southern Denmark, Odense University Hospital, Odense C, Denmark

Agustín Lahoz Unidad de Hepatología Experimental, Unidad Analítica, Instituto de Investigación Sanitaria, Fundación Hospital La Fe, Valencia, Spain

Seow Chong Lee Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Diana J. Leeming Nordic Bioscience, Biomarkers and Research, Herlev, Denmark

Lisiane Lopes da Conceição Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Alberto Lue Liver Transplantation Unit, Gastroenterology and Hepatology Department, University Hospital Lozano Blesa, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain

Philipp Lutz Department of Internal Medicine I, University of Bonn, Bonn, Germany

Timothy J. Lyons Centre for Experimental Medicine, Queen's University of Belfast, Northern Ireland, UK

Manoela Maciel dos Santos Dias Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Pamela Maffioli Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

PhD School in Experimental Medicine, University of Pavia, Pavia, Italy

Ki M. Mak Department of Medical Education/Center for Anatomy and Functional Morphology, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Santiago Marfà Biochemistry and Molecular Genetics Service, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

Cláudio Augusto Marroni Post Graduate Program in Medicine: Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil

Elena Martínez-Crespo Liver Transplantation Unit, Gastroenterology and Hepatology Department, University Hospital Lozano Blesa, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain

A. Martini Department of Medicine, University of Padua, Padua, Italy

Barhoum Masad Bar Ilan University, Safed, Israel

Galilee Medical Center, Nahariya, Israel

Mitchell R. McGill Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA

Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA

Giovanni Meli Department of Clinical and Experimental Medicine, University of Catania- Garibaldi Hospital, Catania, Italy

Nahum Méndez-Sánchez Liver Research Unit, Medica Sur Clinic & Foundation, Mexico City, Mexico

Herold J. Metselaar Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands

Daniella Miranda Post Graduate Program in Medicine: Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil

Søren Kragh Moestrup Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark

Holger Jon Møller Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark

Adrianna Mostowska Department of Biochemistry and Molecular Biology, Poznan University of Medical Sciences, Poznań, Poland

Salvatore Musumeci Department of Chemical Sciences, University of Catania and Institute of Biomolecular Chemistry, CNR, Catania, Italy

Valeriya Nemtsova Clinical Pharmacology Department, Kharkov National Medical University, Kharkiv, Ukraine

Marlene Christina Nielsen Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark

Mette J. Nielsen Nordic Bioscience, Biomarkers and Research, Herlev, Denmark

Assy Nimer Department of Internal Medicine A, Galilee Medical Center, Nahariya, Israel

Bar Ilan University, Safed, Israel

Hans Dieter Nischalke Department of Internal Medicine I, University of Bonn, Bonn, Germany

Michael Oellerich Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany

Eugenia Pareja Unidad de Hepatología Experimental, Unidad Analítica, Instituto de Investigación Sanitaria, Fundación Hospital La Fe, Valencia, Spain

Unidad de Cirugía Hepato-Bilio-Pancreatica y Trasplante Hepático, Hospital Universitario y Politécnico La Fe de Valencia, Valencia, Spain

Vinood B. Patel Department of Biomedical Science, Faculty of Science and Technology, University of Westminster, London, UK

Julio Isael Pérez-Carreón Laboratorio de Bioquímica y Estructura de Proteínas, Instituto Nacional de Medicina Genómica, Mexico City, Mexico

Chien Yi M. Png Department of Medical Education, Icahn School of Medicine at Mount Sinai, New York, NY, USA

P. Pontisso Department of Medicine, University of Padua, Padua, Italy

Victor R. Preedy Diabetes and Nutritional Sciences Research Division, Faculty of Life Science and Medicine, King's College London, London, UK

Graziella Privitera Department of Clinical and Experimental Medicine, University of Catania- Garibaldi Hospital, Catania, Italy

Francesco Purrello Department of Clinical and Experimental Medicine, University of Catania- Garibaldi Hospital, Catania, Italy

Rajkumar Rajendram Diabetes and Nutritional Sciences Research Division, Faculty of Life Science and Medicine, School of Biomedical and Health Sciences, King's College London, London, UK

Department of Internal Medicine, King Abdulaziz Medical City, National Guard Hospital Affairs, Riyadh, Saudi Arabia

Sidsel Rødgaard-Hansen Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark

Ricardo Sánchez-Rodríguez Laboratorio de Bioquímica y Estructura de Proteínas, Instituto Nacional de Medicina Genómica, Mexico City, Mexico

Thomas D. Sandahl Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark

Sherif Saleh Department of Hepatobiliary Surgery and Liver Transplantation, National Liver Institute, Minufiya University, Minufiya, Egypt

Sabrina Schlesinger Institut für Epidemiologie, Christian-Albrechts-Universität zu Kiel, Kiel, Germany

Department of Epidemiology and Biostatistics, School of Public Health, Imperial College London, London, UK

Ekkehard Schütz Chronix Biomedical GmbH, Göttingen, Germany

M. Trinidad Serrano Liver Transplantation Unit, Gastroenterology and Hepatology Department, University Hospital Lozano Blesa, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain

Yousery E. Sherif Department of Chemistry, Faculty of Science and Arts, Ulla, Taibah University, Medina, Saudi Arabia

Clinical Pharmacology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt

Qiang Shi Division of Systems Biology, National Center for Toxicological Research, The United States Food and Drug Administration, Jefferson, AR, USA

Estela Solanas Digestive Pathology Group, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain

Peipei Song Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Luisa Spadaro Department of Clinical and Experimental Medicine, University of Catania- Garibaldi Hospital, Catania, Italy

Ulrich Spengler Department of Internal Medicine I, University of Bonn, Bonn, Germany

Marta Stelmach-Mardas Department of Epidemiology, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany

Department of Pediatric Gastroenterology and Metabolic Diseases, Poznan University of Medical Sciences, Poznan, Poland

Scott H. Stewart Department of Medicine, University at Buffalo, State University of New York, Buffalo, NY, USA

Hwee Tong Tan Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Wei Tang Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Ka-Fai To Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Shatin, NT, Hong Kong

Julia Esperanza Torres-Mena Laboratorio de Bioquímica y Estructura de Proteínas, Instituto Nacional de Medicina Genómica, Mexico City, Mexico

Misael Uribe Liver Research Unit, Medica Sur Clinic & Foundation, Mexico City, Mexico

Luc J. W. van der Laan Department of Surgery, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands

Cornelia J. Verhoeven Department of Surgery, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands

Wim Verlinden Department of Gastroenterology and Hepatology, Antwerp University Hospital, Edegem, Belgium

Laboratory of Experimental Medicine and Pediatrics, University of Antwerp, Wilrijk, Belgium

Hendrik Vilstrup Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark

Libor Vítek 4th Department of Internal Medicine, and Institute of Medical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

Luisa Vonghia Department of Gastroenterology and Hepatology, Antwerp University Hospital, Edegem, Belgium

Philip D. Walson Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany

James L. Weemhoff Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA

Benjamin L. Woolbright Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA

Simon C. H. Yu Department of Imaging and Interventional Radiology, The Chinese University of Hong Kong, Shatin, NT, Hong Kong

Cihan Yurdaydin Department of Gastroenterology, University of Ankara Medical School, Ankara, Turkey

Part I

General Aspects and Introductory Material

Traditional Markers in Liver Disease

1

Giuseppe Derosa and Pamela Maffioli

Contents

G. Derosa (✉)
Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for the Study of Endocrine-Metabolic Pathophysiology and Clinical Research, University of Pavia, Pavia, Italy

Laboratory of Molecular Medicine, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
e-mail: giuseppe.derosa@unipv.it

P. Maffioli
Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

PhD School in Experimental Medicine, University of Pavia, Pavia, Italy
e-mail: pamelamaffioli@hotmail.it

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_13

Abstract

Liver diseases are many and can often be difficult to diagnose, because symptoms can be vague and easily confused with other health problems. However, physicians can be helped by specific markers used to diagnose and follow up liver diseases. In fact some of the enzymes and the end products of the metabolic pathway occurring in the liver are very sensitive for the abnormality occurred and, for this reason, may be considered as biochemical markers of liver dysfunction. In this chapter, we will examine the main markers of liver diseases, dividing them as markers of hepatic necrosis, markers of hepatic obstruction, markers of liver's biosynthetic capacity, markers of hepatic steatosis, markers of hepatic fibrosis, and markers of hepatic tumor.

Keywords

Biosynthesis • Fibrosis • Liver diseases • Markers • Necrosis • Steatosis

List of Abbreviations

AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
LDH	Lactate dehydrogenase
γ-GT	Gamma glutamyl transferase
PT	Prothrombin time
INR	International normalized ratio
PICP	Procollagen type I carboxy-terminal peptide
PIIINP	Procollagen type III amino-terminal peptide
TGF-β1	Transforming growth factor-β1
AFP	α-Fetoprotein
NTP	5′ Nucleotidase
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis

Key Facts of Liver Diseases

- Liver diseases can be inherited or acquired, caused by a variety of factors including viral infections; illegal drug use; legal drug overuse, in particular paracetamol or acetaminophen; and alcohol abuse. Obesity is also associated with liver damage.
- There are over a hundred different forms of liver disease that affect both sexes at different ages.
- Liver disease can often be difficult to diagnose, because symptoms can be vague and easily confused with other health problems. All these conditions have specific markers used to diagnose and follow up them.
- The knowledge of biomarkers linked to liver disease is very important to promptly diagnose liver abnormalities and to guide physicians in the right direction to identify the causes.

Introduction

The liver is a vital organ, involved in several metabolic processes such as metabolism of fats, sugars, proteins, and vitamins and in the regulation of blood clotting. The liver plays the main role in the body's defenses, filtering toxins and microbes from the blood and regulating processes activated in responses to trauma, stress, or inflammation. The liver is also able to regenerate and repair itself. Given that the liver is such a complex organ, performing over 500 functions, it is not surprising that its function can be damaged in several ways. Liver diseases can be inherited or acquired, caused by a variety of factors including viral infections; illegal drug use; legal drug overuse, in particular paracetamol or acetaminophen; and alcohol abuse. Obesity is also associated with liver damage. Over time, damage to the liver results in cirrhosis, which can lead to liver failure, a life-threatening condition. There are over a hundred different forms of liver disease that affect both sexes at different ages. Liver diseases include: Alagille syndrome, alpha-1 antitrypsin deficiency, autoimmune hepatitis, biliary atresia, cirrhosis, cystic disease, fatty liver disease, galactosemia, gallstones, Gilbert's syndrome, hemochromatosis, liver cancer, lysosomal acid lipase deficiency, neonatal hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, porphyria, Reye's syndrome, sarcoidosis, toxic hepatitis, type 1 glycogen storage disease, tyrosinemia, Wilson's disease, and viral hepatitis A, B, and C.

Liver disease can often be difficult to diagnose, because symptoms can be vague and easily confused with other health problems. All these conditions have specific markers used to diagnose and follow up them. Some of the enzymes and the end products of the metabolic pathway occurring in the liver are very sensitive for the abnormality occurred and, for this reason, may be considered as biochemical markers of liver dysfunction. In particular, we chose to divide markers of liver disease in different groups, including:

- **Markers of hepatic necrosis**: aminotransferases including aspartate aminotransferase (AST), alanine amino transferase (ALT), and lactate dehydrogenase (LDH)
- **Markers of hepatic obstruction**: conjugated and unconjugated bilirubin, alkaline phosphatase, gamma glutamyl transferase (γ-GT)
- **Markers of liver's biosynthetic capacity**: albumin, ceruloplasmin, α-1 antitrypsin, prothrombin time (PT) and INR, pseudocholinesterase
- **Markers of hepatic steatosis**: alanine amino transferase (ALT), ferritin, ultrasound score of steatosis
- **Markers of hepatic fibrosis**: procollagen type I carboxy-terminal peptide (PICP) and procollagen type III amino-terminal peptide (PIIINP), AST/ALT ratio, constituents of extracellular matrix (hyaluronic acid, type IV collagen 7S domain, TGF-β1, metalloproteinase), FibroScan scoring
- **Markers of hepatic tumor**: α-fetoprotein (AFP), 5′ nucleotidase (NTP)

In this regard, the aim of this chapter will be to examine traditional markers in liver disease in order to give readers a guide about diagnosis and follow-up of this kind of disease.

Markers of Hepatic Necrosis

Aminotransferases

The aminotransferases are the most frequently utilized and specific indicators of hepatocellular necrosis, because they are released into the bloodstream from damaged hepatocytes. They belong to a group of enzymes that catalyze the interconversion of amino acids and oxoacids by transfer of amino groups. Aminotransferases include aspartate aminotransferase (AST), also known as serum glutamic oxaloacetic transaminase (SGOT), and alanine aminotransferase (ALT), also known as serum glutamic pyruvic transaminase (SGPT). Alanine aminotransferase is primarily localized into the liver, while AST is present in a wide variety of tissues. Whereas the AST is present in both the mitochondria and cytosol of hepatocytes, ALT is localized to the cytosol (Sherlock 1997). The cytosolic and mitochondrial forms of AST are true isoenzymes and immunologically distinct. About 80% of AST activity in human liver is contributed by the mitochondrial isoenzyme, whereas most of the circulating AST activity in normal people is derived from the cytosolic isoenzyme. Large increases in mitochondrial AST occur in serum after extensive tissue necrosis. Their activity in serum at any moment reflects the relative rate at which they enter and leave circulation. Of the numerous methods used for measuring their levels, the most specific method couples the formation of pyruvate and oxaloacetate – the products of the aminotransferase reactions to their enzymatic reduction to lactate and malate (Nalpas et al. 1986; Rej 1985). The primary clinical application of serum AST and ALT measurement is the detection and differential etiologic diagnosis of hepatic disease. Comparable elevations of both AST and ALT are typical of acute viral, toxic, or non ethanol drug-induced hepatitis. The similar

serum transaminase levels are due to cellular release of only cytoplasmic enzymes associated with reversible hepatic cell damage. In chronic hepatitis and cirrhosis, serum AST levels are higher than ALT, due to hepatic cell necrosis with release of mitochondrial AST. In alcohol hepatitis, AST is more significantly increased than ALT, while in hepatic steatosis, ALT is higher than AST. In this regard, ALT has been used as a surrogate marker for liver fat accumulation, as previously reported (Nanji et al. 1986). Previously reported biochemical studies suggested the existence of two isoforms of ALT in humans – a first isoform called ALT1 located on human chromosome 8q24.3 (Sohocki et al. 1997) and a second isoform, called ALT2, mapped to the human chromosome 16q12.1, which was mainly expressed in muscle and adipose tissues (Yang et al. 2002). An elevated ALT is considered a consequence of hepatocyte damage due to NAFLD. However, the measured plasma elevations of ALT may also be a consequence of high systemic ALT2 isoform levels that are largely derived from adipose tissue, as in obesity and insulin resistance in mice (Jadhao et al. 2004). These are due to insulin resistance, increased pro-inflammatory cytokine production, oxidative stress, and mitochondrial dysfunction leading to hepatocyte damage/destruction (Day 2002).

Lactate Dehydrogenase

Lactate dehydrogenase (LDH) is an enzyme that catalyzes the conversion of lactate to pyruvate, an important step in energy production in cells. Many different types of cells contain LDH, including heart, kidney, liver, and muscle. Lactate dehydrogenase is a cytosolic enzyme which level increases in hepatocellular damage. However, estimation on total LDH activity is of no use as a part of liver function tests, because of the influence of extrahepatic diseases on the total LDH activity. LDH, in fact, is increased in several kinds of cancer, including testicular cancer, Ewing's sarcoma, non-Hodgkin's lymphoma, and some types of leukemia. Moreover, elevated LDH levels can be found in several noncancerous conditions, including heart failure, hypothyroidism, anemia, and lung or liver disease. There are five isoforms of LDH; the LDH_5 predominates in the liver; however, for the reasons reported above, this one is not routinely used in favor of more reliable markers of liver damage (Thapa and Anuj 2007).

Markers of Hepatic Obstruction

Bilirubin

Bilirubin is the catabolic product of hemoglobin produced within the reticuloendothelial system; red cell hemoglobin accounts for approximately 85% of all bilirubin. Bilirubin is released in unconjugated form (indirect bilirubin) which is bound to albumin in the plasma and transported to the liver. Bilirubin is conjugated with glucuronic acid in the hepatocytes; the conjugation is catalyzed by glucuronyl

transferase. Conjugated bilirubin (direct bilirubin) is secreted into the bile and enters the duodenum. In the small bowel, some of the bilirubin is hydrolyzed to yield unconjugated bilirubin and glucuronic acid, with the production of urobilinogen. Most urobilinogen is excreted in the stool, but some is reabsorbed and returned to the liver via the portal vein, enters circulation, and is excreted by the kidney (Nicoll 2007). Normal serum total bilirubin ranges from 2 to 21 μmol/L, unconjugated bilirubin level is less than 12 μmol/L, and conjugated bilirubin is less than 8 μmol/L. Serum bilirubin levels higher than 17 μmol/L suggest liver diseases, and levels higher than 24 μmol/L indicate abnormal laboratory liver tests (Thapa and Anuj 2007; Wong et al. 2004).

Jaundice occurs for bilirubin concentration of 40 μmol/L; at this level, bilirubin becomes visible within the sclera, skin, and mucous membranes (Beckingham and Ryder 2001). Unconjugated bilirubin is insoluble and is not excreted in the urine, but it is liposoluble and can accumulate in the brain and nerve tissues, causing damages; conjugated bilirubin, instead, is soluble and is excreted in the urine. Hyperbilirubinemia can be due to overproduction/impaired uptake, conjugation, or excretion/regurgitation of unconjugated or conjugated bilirubin from hepatocytes to bile ducts. On this basis, jaundice can be classified as prehepatic, hepatic, or posthepatic, and the prevalence of unconjugated or conjugated bilirubin can help to better define jaundice origin (Beckingham and Ryder 2001).

In particular, in prehepatic jaundice there is an excess of unconjugated hyperbilirubinemia. Prehepatic jaundice occurs in situations where unconjugated bilirubin is produced faster than the liver is able to conjugate it for excretion, as in increased hemolysis during spherocytosis, homozygous sickle cell disease, thalassemia major, and reabsorption of large hematomas.

In hepatic jaundice, usually both unconjugated and conjugated bilirubin rise, because necrosis of hepatocytes frees the already conjugated bilirubin and makes them unable to conjugate indirect bilirubin. The most common causes of hepatic jaundice are viral hepatitis, alcoholic cirrhosis, primary biliary cirrhosis, drug-induced jaundice, and alcoholic hepatitis. Also genetic defects can be responsible for hepatic jaundice (Tiribelli and Ostrow 1996). In particular in Gilbert's syndrome and Crigler-Najjar syndrome, there is a defect in the gene that encodes for glucuronyl transferase, which results in a reduction in the liver's ability to conjugate bilirubin; in this condition unconjugated bilirubin is high. In Dubin-Johnson syndrome, instead, there is a defect of ABCC2 gene, involved in the production of a protein called multidrug resistance protein 2 (MRP2) responsible to transport substances out of the liver. In this case, conjugated bilirubin is high.

Finally, posthepatic jaundice is characterized by an excess of conjugated hyperbilirubinemia. It is most often due to biliary obstruction by a stone in the common bile duct or by carcinoma of the pancreas. Pancreatic pseudocyst, chronic pancreatitis, sclerosing cholangitis, a bile duct stricture, or parasites in the bile duct are less common causes (Beckingham and Ryder 2001) (Table 2).

Alkaline Phosphatase

Alkaline phosphatase (ALP) is present in epithelial mucosa of the small intestine, proximal convoluted tubules of the kidney, bone, liver, and placenta. It is involved in lipid transportation in the intestine and calcification in the bones. The serum ALP activity is mainly from the liver, with 50% contributed by the bone (Mauro et al. 2006). Normal serum ALP ranges between 41 and 133 U/L (Nicoll 2007). In acute viral hepatitis, ALP usually remains normal or moderately increased. In the liver, epithelial cells lining the bile canaliculi produce alkaline phosphatase, and its serum activity is raised in patients with intrahepatic cholestasis, cholangitis, or extrahepatic obstruction; increased activity may also occur in patients with focal hepatic lesions in the absence of jaundice. Hepatic and bony metastasis can also be responsible for elevated levels of ALP. Other causes of elevated ALP include infiltrative liver diseases, abscesses, granulomatous liver disease, and amyloidosis. Finally, mildly elevated levels of ALP may be seen in cirrhosis, hepatitis, and congestive cardiac failure (Rosalki and Mcintyre 1999). Alkaline phosphatase is elevated in peripheral arterial disease, independent of other traditional cardiovascular risk factors (Cheung et al. 2009). On the other hand, low levels of ALP occur in hypothyroidism, pernicious anemia, zinc deficiency, and congenital hypophosphatasia (Simko 1991). In the presence of elevated ALP levels, clinicians need to differentiate among liver and bone disorders; in this case, levels of gamma glutamyl transferase (γ-GT) can be helpful, because they will be high in cholestatic disorders and normal in bone diseases (Mauro et al. 2006).

Gamma Glutamyl Transferase

Gamma glutamyl transferase is a microsomal enzyme present in hepatocytes and biliary epithelial cells, renal tubules, pancreas, and intestine. Serum γ-GT activity is mainly linked to hepatobiliary system, even though it is found in more concentration in the renal tissue (Mauro et al. 2006). The normal level of γ-GT is between 9 and 85 U/L (Nicoll 2007). Elevated levels of γ-GT can be found during acute viral hepatitis, the peak occurs in the second or third week of illness, and in some patients remain elevated for 6 weeks (Rosalki and Mcintyre 1999). Also in 30% of patients with chronic hepatitis C infection, γ-GT are elevated (Giannini et al. 2001). Also uncomplicated diabetes mellitus, acute pancreatitis, myocardial infarction, anorexia, Guillain-Barré syndrome, hyperthyroidism, obesity, and myotonic dystrophy can be responsible for elevated levels of γ-GT (Rosalki and Mcintyre 1999). In alcohol abuse, serum γ-GT levels can increase to more than ten times. This is partly due to structural liver damage, hepatic microsomal enzyme induction, or alcoholic pancreatic damage (Wu et al. 1976). Gamma glutamyl transferase can also be an early marker of oxidative stress, since serum antioxidant carotenoids including lycopene, α-carotene, β-carotene, and β-cryptoxanthin are

inversely associated with alcohol-induced increase of serum γ-GT as reported in moderate and heavy drinkers (Sugiura et al. 2005). Another condition responsible for γ-GT levels two to three times higher than the upper reference value in more than 50% of the patients is nonalcoholic fatty liver disease (McCullough 2002). Previously published papers showed a significant positive correlation between serum γ-GT and triglyceride levels in diabetic patients. As reported above, the main use of γ-GT is to help discriminate hepatic diseases from bone diseases when ALP is elevated.

Markers of Liver's Biosynthetic Capacity

Albumin

The liver is responsible for the production of several serum proteins. In particular, parenchymal cells are responsible for synthesis of albumin, fibrinogen, and other coagulation factors and most of the alpha and beta globulins. Albumin is quantitatively the most important plasma protein synthesized by the liver and, for this reason, can be a useful indicator of hepatic function. Albumin half-life in serum is as long as 20 days, and it takes at least 10 days for the concentration to fall below the normal range despite impaired liver function; for this reason, serum albumin level is not a reliable indicator of hepatic protein synthesis in acute liver disease. Even if the liver is the only site of synthesis of albumin, albumin synthesis can be affected also by nutritional status, hormonal balance, and osmotic pressure. Serum albumin levels are typically depressed in patients with cirrhosis and ascites. In patients with or without ascites, the serum albumin level correlates with prognosis, and are included in the Child-Pugh score, a scoring system used to quantify the severity of chronic liver disease inclusive of cirrhosis. The score is composed of five categories, including total bilirubin, serum albumin, INR, the presence of ascites, and the presence of hepatic encephalopathy. The higher the score, the worse the severity of cirrhosis (Table 1).

Normally serum albumin levels range between 3.5 and 4.5 g/dL. The average adult has approximately 300–500 g of albumin. The serum levels at any time reflect its rate of synthesis, degradation, and volume of distribution (Nicoll 2007).

Ceruloplasmin

Ceruloplasmin is synthesized in the liver and is an acute phase protein. It binds with copper and serves as a major carrier for copper in the blood. Normal plasma level of ceruloplasmin is 200–600 mg/L (Nicoll 2007). High levels of ceruloplasmin have been found in infections, rheumatoid arthritis, pregnancy, non-Wilson liver disease, and obstructive jaundice. Low levels, instead, have been reported in neonates, Menkes disease, kwashiorkor, marasmus, protein losing enteropathy,

Table 1 Child-Pugh score

Parameter	Value	Score
Total bilirubin	<34 μmol/L	1 point
	34–50 μmol/L	2 points
	>50 μmol/L	3 points
Serum albumin	>3.5 g/dL	1 point
	2.8–3.5 g/dL	2 points
	<2.8 g/dL	3 points
INR	<1.7	1 point
	1.7–2.3	2 points
	>2.3	3 points
Ascites	None	1 point
	Mild	2 points
	Moderate	3 points
Hepatic encephalopathy	None	1 point
	Grades I–II	2 points
	Grades III–IV	3 points
Child-Pugh total score	Class A	5–6 points
	Class B	7–9 points
	Class C	10–15 points

Table 2 Differential diagnosis of jaundice

	Urine		Blood			
	Urobilinogen	Bilirubin	Urobilinogen	Bilirubin	ALT and AST	γ−GT and ALP
Normality	Trace	Absent	Normal	Normal	Normal	Normal
Prehepatic jaundice	Increased	Absent	Increased	Normal	Normal	Normal
Posthepatic jaundice	Decreased or absent	Present	Normal	Increased	Normal or mild increase	Marked increase
Intrahepatic jaundice	Decreased or absent	Present	Increased	Increased	Marked increased	Normal or mild increase

copper deficiency, and aceruloplasminemia (Mauro et al. 2006). In Wilson's disease, a rare inherited disorder that causes too much copper to accumulate in your liver, brain, and other vital organs, ceruloplasmin level is depressed. Decreased rate of synthesis of the ceruloplasmin is responsible for copper accumulation in the liver, because of copper transport defect in Golgi apparatus, since ATP7B is affected (Rosalki and Mcintyre 1999). Serum ceruloplasmin levels were high in the chronic active liver disease (CALD), but low in the Wilson's disease; for this reason, ceruloplasmin levels are the most reliable screening test to differentiate between chronic active liver disease and Wilson's disease (LaRusso et al. 1976).

α-1 Antitrypsin

Alpha-1 antitrypsin (α-1 antitrypsin) is a glycoprotein synthesized by the liver and is an inhibitor of serine proteinases, especially elastase. After being synthesized in the liver, α-1 antitrypsin is normally released in the bloodstream and reaches the lungs. If there are some mutations in the coding sequence of α-1 antitrypsin, its export from the hepatocyte is blocked and it gets stuck in the liver cells. Alpha-1 antitrypsin has many important roles in the lung, including removing bacteria and fighting infections; it is needed to control enzyme activity to prevent healthy lung tissue from being damaged. So a deficiency in the concentration of circulating α-1 antitrypsin predisposes to early onset panlobular emphysema, even in nonsmokers. Moreover, the abnormal accumulation of the glycoprotein in hepatocytes results in programmed cell death, hepatic inflammation, fibrosis, and cirrhosis (Fairbanks, and Tavill 2008). Its normal concentration is 1–1.6 g/L.

Prothrombin Time and INR

Clotting is the final step of a complex series of enzymatic reactions that involve at least 13 factors. The liver is the major site of synthesis of several coagulation factors: I (fibrinogen), II (prothrombin), IV, V, VI, VII, IX, X, and XI. Most of these are present in excess, and abnormalities of coagulation happen when there is substantial impairment in the ability of the liver to synthesize them. This occurs in both biliary obstruction and parenchymal liver disease, because of a combination of poor absorption of fat soluble vitamin K, due to the absence of bile in the gut, and a reduced ability of damaged hepatocytes to produce clotting factors. Abnormal clotting can be measured using prothrombin time of Quick (PT), which evaluates the extrinsic coagulation pathway. It can be expressed in seconds or as a ratio of the plasma prothrombin time to control plasma time (prolonged international normalized ratio or INR), which is also used, as already reported above, to calculate Child-Pugh score (Table 1). Normal value of PT is between 9 and 11 s, a prolongation of more than 2 s is considered abnormal. In acute and chronic hepatocellular disease, the PT may serve as a prognostic indicator (Friedman et al. 2003). However, a prolonged PT is not specific for liver diseases; it can be found in various deficiencies of coagulation factors, disseminated intravascular coagulation, and ingestion of certain drugs.

Pseudocholinesterase

Pseudocholinesterase is primarily synthesized in the liver. When liver function is impaired, pseudocholinesterase synthesis is also impaired. Serum cholinesterase activity can determine if there is a quantitative defect in enzyme function. Pseudocholinesterase deficiency impairs the metabolism of succinylcholine, mivacurium, or

ester local anesthetics. Normal range is between 3,200 and 6,600 IU/L. This number varies by different laboratory standards and is subject to much interindividual variability. Decreased serum activities have been shown in many liver diseases, such as cirrhosis, end-stage liver disease, hepatitis, and liver abscesses. In patients with end-stage liver disease, normal serum pseudocholinesterase levels were again seen after liver transplant, with the transplanted liver assuming the role of production immediately. Additionally, serum cholinesterase activity may drop 30–50% in acute hepatitis, with a 50% decrease in cirrhosis and chronic malignancies being perhaps among some of the most substantial decreases of the acquired conditions. However, as the half-life of serum cholinesterase is approximately 10–14 days, it is considered an unreliable source for tracking liver disease (Soliday et al. 2010).

Markers of Hepatic Steatosis

Liver steatosis or nonalcoholic fatty liver disease (NAFLD) is characterized by a wide spectrum of conditions. NAFLD covers a histological spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis, and cirrhosis. Simple steatosis without fibrosis or inflammation has a benign clinical course in most cases without excess mortality. NASH, instead, may have a more progressive course that can lead to cirrhosis in 10–15% of patients, affecting survival (Ekstedt et al. 2006). It develops in subjects who are not heavy alcohol consumers and have negative tests for viral and autoimmune liver diseases (Angulo 2002; Matteoni et al. 1999; Brunt 2001). Recently, NAFLD has been linked to insulin resistance and type 2 diabetes mellitus, and metabolic syndrome (Cortez-Pinto et al. 1999; Marchesini et al. 2001).

Alanine Aminotransferase

We have already described ALT role as a marker of hepatic steatosis in the paragraph about index of hepatic necrosis.

Ferritin

Increased ferritin, but normal transferrin saturation, is frequently found in patients with hepatic steatosis. The simultaneous disorder of iron and glucose and/or lipid metabolism, in most of the cases associated with insulin resistance, is responsible for persistent hyperferritinemia and identifies patients at risk for NASH (Fargion et al. 2001). Indeed, serum ferritin level was significantly higher in the NASH patients than those with simple steatosis, according to a Japanese study (Yoneda et al. 2010). In that study, the serum ferritin level was related with insulin resistance.

Ultrasound Score of Steatosis

Percutaneous liver biopsy is the current standard means of diagnosing and grading steatosis, but it is an invasive procedure with potentially serious complications including hemorrhage, infection, bile leak, and a mortality of up to 0.3% (Bravo et al. 2001). For this reason, noninvasive methods such as computed tomography (CT), magnetic resonance imaging (MRI), and sonography are more commonly applied in clinical practice and in population-based studies (Joy et al. 2003; Saadeh et al. 2002; Siegelman and Rosen 2001). The most diffuse method is ultrasonography, because it is cost-effective and widely available, even if it is limited by interobserver and intra-observer variability (Strauss et al. 2007).

For an approximative estimation of hepatic steatosis, hepatic parenchyma can be compared to kidney parenchyma during ultrasound examination (Fig 2): in normal conditions, the liver and renal cortex are of a similar echogenicity; in steatosis, instead, renal cortex appears hypoechoic compared to the liver parenchyma. The brighter the hepatic parenchyma compared to the kidney parenchyma, the higher the steatosis degree. A better grading of severity of hepatic steatosis is possible with an ultrasound score, according to this score:

- Level 0 was defined as a normal hepatic echo pattern.
- Level 1 was defined as a slight increase in echo pattern with normal visualization of vessels and diaphragm.
- Level 2 was defined as a moderate increase in echogenicity with reduced visibility of portal veins and diaphragm.
- Level 3 was defined as a pronounced increase in hepatic echo pattern with poor visibility of intrahepatic vessels and posterior right lobe of the liver.

This score derives from the evaluation of different aspects of the liver during ultrasound examination that considers liver echotexture, echo penetration and visibility of diaphragm, and clarity of liver blood vessel structures (Chan et al. 2004).

Markers of Hepatic Fibrosis

Procollagen Type I Carboxy-Terminal Peptide (PICP) and Procollagen Type III Amino-Terminal Peptide (PIIINP)

In the healthy human liver, the most abundant collagens are the fibril-forming types I and III. Collagen types I and III are synthesized as procollagens with a small amino-terminal and a larger carboxy-terminal propeptide. Once secreted into the extracellular space, the propeptides are removed by specific endopeptidases, thus allowing integration of the rigid collagen triple helix into the growing fibril. After this process type I carboxy-terminal peptide (PICP) is cleaved off procollagen type I during synthesis of the fibril-forming collagen type I, while the three-amino acid

procollagen type III amino-terminal peptide (PIIINP) is cleaved off procollagen type III forming collagen type III (Nimni 1993). Both are released in serum, and for this reason their serum concentrations have been proposed as a useful marker of collagen type I and III synthesis (Veidal et al. 2010). This is supported by a diversity of clinical observations demonstrating that high serum levels of these peptides reflect ongoing tissue fibrosis. During fibrogenesis, type I collagen levels increase up to eightfold. Additionally, the ratio of the type I/III also changes from 1:1 in the healthy liver to 1:2 in the cirrhotic liver (Sakugawa et al. 2005).

PICP levels are normal in patients with mild chronic hepatitis C and elevated in 50% of patients with moderately advanced or advanced chronic hepatitis C, including patients with liver cirrhosis of this etiology. PIIINP relative concentration in the basement membrane is higher in hepatic fibrogenesis (Lieber et al. 2008). In acute hepatitis, levels of serum PIIINP correlate with aminotransferase levels. In chronic liver disease, serum PIIINP reflects the stage of liver fibrosis (Giboney 2005). Unfortunately, PIIINP is not specific for the fibrosis of the liver as it is also elevated in acromegaly, lung fibrosis, chronic pancreatitis, and rheumatologic disease (Sakugawa et al. 2005).

AST/ALT Ratio

The predictive value of the AST/ALT ratio has been validated in nonalcoholic liver disease, chronic viral hepatitis, primary sclerosing cholangitis, and primary biliary cirrhosis (Lieber et al. 2008). In many forms of acute and chronic liver injury or steatosis (fatty infiltration of the liver), this ratio is less than or equal to 1, while in alcoholic hepatitis, an AST/ALT ratio is often greater than 2.

Constituents of Extracellular Matrix

The constituents of extracellular matrix are expected to be released into circulation during turnover of fibrosis in the liver. For this reason, they are reliable markers to differentiate NASH from simple steatosis, especially those with significant liver fibrosis. Marked elevation of serum hyaluronic acid and type IV collagen 7S domain, both extracellular matrix components, occurred in NASH patients with advanced fibrosis compared to those with mild fibrosis. Serum hyaluronic acid levels were also markedly elevated in patients with NASH than with steatosis only. Hyaluronic acid is a high molecular weight glycosaminoglycan, which is an essential component of extracellular matrix in virtually every tissue in the body. In the liver, it is synthesized by the hepatic stellate cells and degraded by the sinusoidal endothelial cells (Lindqvist, and Laurent 1992). The best cutoff value using ROC analysis was $\geq$43 ng/mL to detect NASH, and $\geq$50 ng/mL to detect severe fibrosis (Yoneda et al. 2007). Regarding type IV collagen 7S domain, it is involved in connective tissue metabolism, and has been identified as a biochemical marker for assessing fibrogenesis and the severity of fibrosis in patients with cirrhosis (Yoneda et al. 2007).

The best cutoff value using ROC analysis was ≥5 ng/mL to detect NASH and ≥5 ng/mL to detect severe fibrosis for type IV collagen 7S domain. The positive predictive value (PPV) for detecting NASH can be as high as 97.1% when both markers are greater than the cutoffs.

Another marker of hepatic fibrosis is transforming growth factor-β1 (TGF-β1), a cytokine involved in tissue growth, differentiation, extracellular matrix production, and immune response. This cytokine has three isoforms (β1, β2, and β3), but only TGF-β1 has been linked to liver fibrogenesis. A correlation between TGF-β1 levels and the rate of fibrosis progression has been reported (Kanzler et al. 2001).

Other constituents of extracellular matrix are metalloproteinases (MMPs); they belong to a family of structurally related proteolytic enzymes that mediate the degradation of the extracellular matrix and the basal membranes (Sun 2010). The three most commonly studied human metalloproteinases are MMP-2 (gelatinase-A), MMP-3 (stromelysin), and MMP-9 (gelatinase-B). MMP-2 is secreted by activated hematopoietic stem cells; elevated levels of MMP-2 and its proenzyme have been observed in various liver diseases (Takahara et al. 1997). During hepatic fibrogenesis, the expression of MMP-2 is markedly increased. The potential for MMP-2 for predicting liver fibrosis remains unclear as some contradictory data have been reported by previous studies (Walsh et al. 1999; Hayasaka et al. 1996). In contrast to MMP-2, MMP-9 levels show their value mainly in the diagnosis of hepatocellular carcinoma (Badra et al. 2010): MMP-9 levels were negatively correlated to the histological severity of the liver disease in patients with chronic hepatitis C. Metalloproteinases activity is strictly controlled by tissue inhibitors of matrix metalloproteinases (TIMPs); TIMPs are secreted proteins that interact with MMPs and modulate their activation and functioning. TIMP-1 controls activity of most MMPs, whereas TIMP-2 specifically inhibits MMP-2. TIMPs-dependent inhibition of extracellular matrix degradation may promote liver fibrosis; elevation of TIMPs' levels has been observed in chronic liver disease.

FibroScan Scoring Card

Hepatic fibrosis can be quantified throughout assessment of stiffness, using an ultrasound-based technology introduced in the latest years. This technique called transient ultrasound elastography or FibroScan measures the stiffness of the hepatic parenchyma using both ultrasound (5 MHz) and low-frequency (50 Hz) elastic waves produced by a specialized ultrasound vibrator applied to the body wall and coupled with 1D ultrasound imaging that measures the propagation speed of a wave using a pulse-echo ultrasound. Since fibrotic tissue is harder than healthy liver tissue, the shear wave measurement provides immediate quantitative assessment of the degree of stiffness. FibroScan was reported to be a reliable method for the diagnosis of significant fibrosis (AUC = 0.84), severe fibrosis (AUC = 0.89), and cirrhosis (AUC = 0.94) accompanying various liver diseases including hepatitis B and C, alcoholic liver disease, and nonalcoholic fatty liver disease (NAFLD) (Ziol et al. 2005; Friedrich-Rust et al. 2008). However, previously

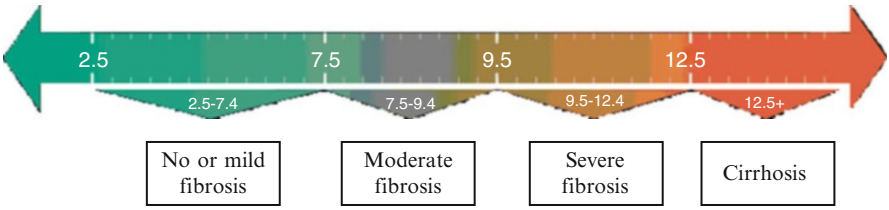

Fig. 1 FibroScan score and fibrosis degree

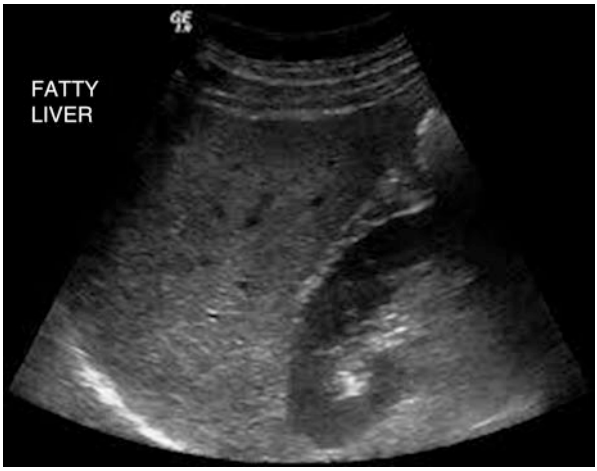

Fig. 2 Ultrasound image of hepatic steatosis

reported papers showed that FibroScan accuracy in assessing lower degrees of liver fibrosis is not as reliable compared to diagnosing advanced fibrosis and cirrhosis (Ziol et al. 2005).

Stiffness assessed by FibroScan is expressed in kPa, using a score between 2.5 and 75 kPa. Between 90 and 95% of healthy people without liver disease will have a liver scarring measurement less than 7.0 kPa; patients with chronic hepatitis C and a liver stiffness more than 14 kPa has approximately a 90% probability of having cirrhosis, while patients with liver stiffness more than 7 kPa have around an 85% probability of at least significant fibrosis (Fig. 1).

Markers of Hepatic Tumor

α-Fetoprotein

The α-fetoprotein (AFP) gene is highly activated in the fetal liver, but is significantly repressed shortly after birth. The normal level of AFP is 0–15 μg/L (Nicoll 2007). The finding of elevated AFP levels in response to liver injury and during the early

stages of chemical hepatocarcinogenesis led to the conclusion that maturation arrest of liver-determined tissue stem cells gives rise to hepatocellular carcinomas. An AFP value above 400–500 μg/L has been considered to be diagnostic for hepatocellular carcinoma (HCC) in patients with cirrhosis. A higher AFP concentration, ≥400 μg/L in HCC patients, is associated with greater tumor size, bilobar involvement, portal vein invasion, and a lower median survival rate (Gowda et al. 2009). It has also been reported that higher serum AFP levels independently predict a lower sustained virological response rate among patients with chronic hepatitis C. There are three different AFP variants, differing in their sugar chains (AFP-L1, AFP-L2, AFP-L3). AFP-L1 is the main glycoform of AFP in the serum of patients with nonmalignant chronic liver disease, while AFP-L3 is the main glycoform of AFP in the serum of HCC patients. α-Fetoprotein-L3 can be detected in one-third of patients with small HCC (<3 cm); it acts as a marker for clearance of HCC after treatment: an AFP-L3 level of 15% or more is correlated with HCC portal vein invasion (Hagiwara et al. 2006). Estimating the AFP-L3/AFP ratio can be helpful in diagnosis and prognosis of HCC (Asmaa et al. 2009).

5′ Nucleotidase

5′ Nucleotidase is a glycoprotein generally disseminated throughout the tissues of the body; it is localized in cytoplasmic membrane, and it catalyzes the release of inorganic phosphate from nucleoside-5′-phosphates. The normal range established is 0–15 U/L (Nicoll 2007). Raised levels of NTP activity were found in patients with obstructive jaundice, parenchymal liver disease, hepatic metastases, and bone disease (Daniel and Marshal 2007). Elevation of NTP can be found in acute infective hepatitis and in chronic hepatitis (Pratibha et al. 2004). However, NTP is almost a marker of early hepatic primary or secondary tumors. The increase of both ALP and NTP suggests intra- or extrahepatic obstruction due to malignancy (Smith et al. 1966).

Potential Applications to Prognosis, Other Diseases, or Conditions

Liver diseases have specific markers that can be used to diagnose and follow up them. Some of the enzymes and the end products of the metabolic pathway occurring in the liver are very sensitive for the abnormality occurred and, for this reason, may be considered as biochemical markers of liver dysfunction. The knowledge of biomarkers linked to liver disease is very important to promptly diagnose liver abnormalities and to guide physicians in the right direction to identify the causes. This can help to reduce the costs linked to liver diseases, their diagnosis and treatment.

Summary Points

- This chapter focuses on biomarkers relevant to liver diseases.
- Biomarkers include measurable indicators of some biological state and are useful to diagnose or follow up a specific condition or risk factor.
- Biomarkers relevant to liver diseases include markers of hepatic necrosis, hepatic obstruction, liver's biosynthetic capacity, hepatic steatosis, hepatic fibrosis, and hepatic tumor.
- The knowledge of biomarkers linked to liver disease is very important to promptly diagnose liver abnormalities and to guide physicians in the right direction to identify the causes.

Mini Dictionary

Biomarker: the term refers to a measurable indicator of some biological state or condition that can be used for diagnosis or follow-up of a particular disease.

Hepatic necrosis: the term refers to death of hepatic parenchyma which may involve single cell, or multicell in piecemeal, focal, periacinar, midzonal, periportal, or paracentral locations.

Biosynthetic capacity: the term refers to the production of a complex chemical compound from simpler precursors in a living organism.

Hepatic steatosis: the term refers to excessive amounts of triglycerides and other fats inside liver cells.

Hepatic fibrosis: the term refers to a reaction to chronic injury to the liver; it includes biliary fibrosis, postnecrotic scarring, diffuse hepatic fibrosis, and periacinar fibrosis.

References

Angulo P. Nonalcoholic fatty liver disease. N Engl J Med. 2002;346:1221–31.

Asmaa IG, Shahid AK, Edward LS. Diagnosis of hepatocellular carcinoma. World J Gastroenterol. 2009;15:1301–14.

Badra G, Lotfy M, El-Refaie A, Obada M, Abdelmonem E, Kandeel S, et al. Significance of serum matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in chronic hepatitis C patients. Acta Microbiol Immunol Hung. 2010;57(1):29–42.

Beckingham IJ, Ryder SD. Clinical review ABC of diseases of liver, pancreas, and biliary system investigation of liver and biliary disease. BMJ. 2001;322:33–6.

Bravo AA, Sheth SG, Chopra S. Liver biopsy. N Engl J Med. 2001;344:495–500.

Brunt EM. Nonalcoholic steatohepatitis: definition and pathology. Semin Liver Dis. 2001;21:3–16.

Chan DF, Li AM, Chu WC, Chan MH, Wong EM, Liu EK, et al. Hepatic steatosis in obese Chinese children. Int J Obes Relat Metab Disord. 2004;28(10):1257–63.

Cheung BM, Ong KL, Wong LY. Elevated serum alkaline phosphatase and peripheral arterial disease in the United States national health and nutrition examination survey 1999–2004. Int J Cardiol. 2009;135:156–61.

Cortez-Pinto H, Camilo ME, Baptista A, De Oliveira AG, De Moura MC. Non-alcoholic fatty liver: another feature of the metabolic syndrome? Clin Nutr. 1999;18:353–8.
Daniel SP, Marshal MP. In: Eugene RS, Michel FS, Willis CM, editors. Schiff's diseases of the liver, vol. 1. 10th ed. Philadelphia: Lippincott Williams and Wilkins; 2007. p. 19–54. Laboratory test.
Day CP. Pathogenesis of steatohepatitis. Best Pract Res Clin Gastroenterol. 2002;16:663–78.
Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology. 2006;44:865–73.
Fairbanks KD, Tavill AS. Liver disease in alpha 1-antitrypsin deficiency: a review. Am J Gastroenterol. 2008;103(8):2136–41.
Fargion S, Mattioli M, Fracanzani AL, Sampietro M, Tavazzi D, Fociani P, et al. Hyperferritinemia, iron overload, and multiple metabolic alterations identify patients at risk for nonalcoholic steatohepatitis. Am J Gastroenterol. 2001;96:2448–55.
Friedman SF, Martin P, Munoz JS. Laboratory evaluation of the patient with liver disease. Hepatology, a textbook of liver disease. Philedelphia: Saunders; 2003. p. 661–709.
Friedrich-Rust M, Ong MF, Martens S, Sarrazin C, Bojunga J, Zeuzem S, et al. Performance of transient elastography for the staging of liver fibrosis: a meta-analysis. Gastroenterology. 2008;134(4):960–74.
Giannini E, Botta F, Fasoli A, Romagnoli P, Mastracci L, Ceppa P. Increased levels of gamma GGT suggest the presence of bile duct lesions in patients with chronic hepatitis C: absence of influence of HCV genotype, HCV-RNA serum levels, and HGV infection on this histological damage. Dig Dis Sci. 2001;46:524–9.
Giboney PT. Mildly elevated liver transaminase levels in the asymptomatic patient. Am Fam Physician. 2005;71(6):1105–10.
Gowda S, Desai PB, Hull VV, Math AA, Vernekar SN, Kulkarni SS. A review on laboratory liver function tests. Pan Afr Med J. 2009;3:17.
Hagiwara S, Kudo M, Kawasaki T, Nagashima M, Minami Y, Chung H, et al. Prognostic factors for portal venous invasion in patients with hepatocellular carcinoma. J Gastroenterol. 2006;41:1214–9.
Hayasaka A, Suzuki N, Fujimoto N, Iwama S, Fukuyama E, Kanda Y, et al. Elevated plasma levels of matrix metalloproteinase-9 (92-kd type IV collagenase/gelatinase B) in hepatocellular carcinoma. Hepatology. 1996;24:1058–62.
Jadhao SB, Yang RZ, Lin Q, Hu H, Anania FA, Shuldiner AR, Gong DW. Murine alanine aminotransferase: cDNA cloning, functional expression, and differential gene regulation in mouse fatty liver. Hepatology. 2004;39:1297–302.
Joy D, Thava VR, Scott BB. Diagnosis of fatty liver disease: is biopsy necessary? Eur J Gastroenterol Hepatol. 2003;15:539–43.
Kanzler S, Baumann M, Schirmacher P, Dries V, Bayer E, Gerken G, et al. Prediction of progressive liver fibrosis in hepatitis C infection by serum and tissue levels of transforming growth factor beta. J Viral Hepat. 2001;8(6):430–7.
LaRusso NF, Summerskill WH, McCall JT. Abnormalities of chemical tests for copper metabolism in chronic active liver disease: differentiation from Wilson's disease. Gastroenterology. 1976;70:653–5.
Lieber CS, Weiss DG, Paronetto F, Veterans Affairs Cooperative Study 391 Group. Value of fibrosis markers for staging liver fibrosis in patients with precirrhotic alcoholic liver disease. Alcohol Clin Exp Res. 2008;32(6):1031–9.
Lindqvist U, Laurent TC. Serum hyaluronan and aminoterminal propeptide of type III procollagen: variation with age. Scand J Clin Lab Invest. 1992;52(7):613–21.
Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, et al. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes. 2001;50:1844–50.
Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology. 1999;116:1413–9.

Mauro P, Renze B, Wouter W. Enzymes. In: Carl AB, Edward R, David EB, editors. Tietz textbook of clinical chemistry and molecular diagnostics. 4th ed. St. Louis: Elsevier; 2006. p. 604–16.

McCullough AJ. Update on nonalcoholic fatty liver disease. J Clin Gastroenterol. 2002;34:255–62.

Nalpas B, Vassault A, Charpin S, Lacour B, Berthelot P. Serum mitochondrial aspartate aminotransferase as a marker of chronic alcoholism: diagnostic value and interpretation in a liver unit. Hepatology. 1986;6(4):608–14.

Nanji AA, French SW, Freeman JB. Serum alanine aminotransferase to aspartate aminotransferase ratio and degree of fatty liver in morbidly obese patients. Enzyme. 1986;36:266–9.

Nicoll DC. In: Stephen JM, Maxine AP, editors. Current medical diagnosis and treatment. 46th ed. Columbos: Mc Graw hill; 2007. p. 1767–75. Appendix: Therapeutic drug monitoring and laboratory reference ranges.

Nimni ME. Fibrillar collagens: their biosynthesis, molecular structure, and mode of assembly. In: Zern MA, Reid LM, editors. Extracellular matrix. New York: Marcel Dekker; 1993. p. 121–48.

Pratibha K, Usha A, Rajni A. Serum adenosine deaminase, 5′ nucleotidase and malondialdehyde in acute infective hepatitis. Indian J Clin Biochem. 2004;19:128–31.

Rej R. Measurement of aminotransferases, aspartate aminotransferases. CRC Crit Rev Clin Lab Sci. 1985;21:99–103.

Rosalki SB, Mcintyre N. Biochemical investigations in the management of liver disease, vol. 2. New York: Oxford university press; 1999. p. 503–21. Oxford textbook of clinical hepatology.

Saadeh S, Younossi ZM, Remer EM, Gramlich T, Ong JP, Hurley M, Mullen KD, Cooper JN, Sheridan MJ. The utility of radiological imaging in non-alcoholic fatty liver disease. Gastroenterology. 2002;123:745–50.

Sakugawa H, Nakayoshi T, Kobashigawa K, Yamashiro T, Maeshiro T, Miyagi S, et al. Clinical usefulness of biochemical markers of liver fibrosis in patients with nonalcoholic fatty liver disease. World J Gastroenterol. 2005;11:255–9.

Sherlock S. Assessment of liver function disease of liver and biliary system: Sheila Sherlock. 10th ed. London: Blackwell science ltd; 1997. p. 17–32.

Siegelman ES, Rosen MA. Imaging of hepatic steatosis. Semin Liver Dis. 2001;21:71–80.

Simko V. Alkaline phosphatases in biology and medicine. Dig Dis. 1991;9:189–93.

Smith K, Varon HH, Race GJ, et al. Serum 5′-nucleotidase in patients with tumour in the liver. Cancer. 1966;17:1281–5.

Sohocki MM, Sullivan LS, Harrison WR, Sodergren EJ, Elder FF, Weinstock G. Human glutamate pyruvate transaminase (GPT): localization to 8q24.3, cDNA and genomic sequences, and polymorphic sites. Genomics. 1997;40:247–52.

Soliday FK, Conley YP, Henker R. Pseudocholinesterase deficiency: a comprehensive review of genetic, acquired, and drug influences. AANA J. 2010;78(4):313–20.

Strauss S, Gavish E, Gottlieb P, Katsnelson L. Interobserver and intraobserver variability in the sonographic assessment of fatty liver. AJR. 2007;189(6):W320–3.

Sugiura M, Nakamura M, Ikoma Y, Yano M, Ogawa K, Matsumoto H, et al. High serum carotenoids are inversely associated with serum gamma-glutamyl transferase in alcohol drinkers within normal liver function. J Epidemiol. 2005;15:180–6.

Sun J. Matrix metalloproteinases and tissue inhibitor of metalloproteinases are essential for the inflammatory response in cancer cells. J Signal Transduct. 2010;2010:1–7.

Takahara T, Furui K, Yata Y, Jin B, Zhang LP, Nambu S, et al. Dual expression of matrix protease-2 and membrane type I-matrix proteinase in fibrotic human livers. Hepatology. 1997;26:1521–9.

Thapa BR, Anuj W. Liver function tests and their interpretation. Indian J Pediatr. 2007;74:663–71.

Tiribelli C, Ostrow JD. New concepts in bilirubin and jaundice: report of the third international bilirubin workshop, April 6–8, 1995, Trieste, Italy. Hepatology. 1996;24:1296–311.

Veidal SS, Vassiliadis E, Bay-Jensen AC, Tougas G, Vainer B, Karsdal MA. Procollagen type I N-terminal propeptide (PINP) is a marker for fibrogenesis in bile duct ligation-induced fibrosis in rats. Fibrogenesis Tissue Repair. 2010;3(1):5.

Walsh KM, Timms P, Campbell S, MacSween RN, Morris AJ. Plasma levels of matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases-1 and -2 (TIMP-1 and TIMP-2) as noninvasive markers of liver disease in chronic hepatitis C: comparison using ROC analysis. Dig Dis Sci. 1999;44:624–30.

Wong HY, Tan JYL, Lim CC. Abnormal liver function test in symptomatic pregnant patient: the local experience in Singapore. Ann Acad Med. 2004;33:204–8.

Wu A, Slavin G, Levi AJ. Elevated serum gamma-glutamyl-transferase (transpeptidase) and histological liver damage in alcoholism. Am J Gastroenterol. 1976;65:318–23.

Yang RZ, Blaileanu G, Hansen BC, Shuldiner AR, Gong DW. cDNA cloning, genomic structure, chromosomal mapping, and functional expression of a novel human alanine aminotransferase. Genomics. 2002;79:445–50.

Yoneda M, Mawatari H, Fujita K, Yonemitsu K, Kato S, Takahashi H, Kirikoshi H, Inamori M, Nozaki Y, Abe Y, Kubota K, Saito S, Iwasaki T, Terauchi Y, Togo S, Maeyama S, Nakajima A. Type IV collagen 7s domain is an independent clinical marker of the severity of fibrosis in patients with nonalcoholic steatohepatitis before the cirrhotic stage. J Gastroenterol. 2007;42(5):375–81.

Yoneda M, Nozaki Y, Endo H, Mawatari H, Iida H, Fujita K, et al. Serum ferritin is a clinical biomarker in Japanese patients with nonalcoholic steatohepatitis (NASH) independent of HFE gene mutation. Dig Dis Sci. 2010;55:808–14.

Ziol M, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, de Lédinghen V, et al. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. Hepatology. 2005;41(1):48–54.

Hepascore and Its Application to Liver Disease

2

Yi Huang, Gary P. Jeffrey, and Leon A. Adams

Contents

Abstract

Accurate quantification of the severity of liver fibrosis provides important prognostic information among patients with chronic liver disease and thus influences management. Histopathological staging of liver biopsy is considered the gold standard for assessment of liver fibrosis but is limited by its invasive nature, high cost, and sampling error. Hepascore is a serum model consisting of hyaluronic acid, bilirubin, alpha2-macroglobulin, gamma-glutamyl transpeptidase, age, and

Y. Huang • G.P. Jeffrey • L.A. Adams (✉)
School of Medicine and Pharmacology, University of Western Australia, Perth, WA, Australia
e-mail: yi.huang@uwa.edu.au; gary.jeffrey@uwa.edu.au; leon.adams@uwa.edu.au; leon.adams@health.wa.gov.au

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_29

sex, originally developed to predict the severity of liver fibrosis in patients with chronic hepatitis C. Hepascore has been widely validated in chronic hepatitis C, chronic hepatitis B, alcoholic and nonalcoholic fatty liver disease, and other types of chronic liver disorders. Validation studies have confirmed good to excellent accuracy to predict or exclude different levels of hepatic fibrosis including cirrhosis. In addition, Hepascore is dynamic over time and has been demonstrated to predict adverse clinical outcomes in longitudinal cohorts of chronic hepatitis C and alcoholic liver disease patients. Thus, Hepascore is a valuable clinical tool to stage liver fibrosis and determine prognosis, need for treatment, screening, and surveillance strategies in patients with chronic liver disease.

Keywords

Hepascore • Serum models • Liver fibrosis • Chronic liver disease • Clinical outcomes

List of Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUC	Area under ROC curves
CFLD	Cystic fibrosis liver disease
CI	Confidence interval
CV	Coefficients of variation
GGT	Gamma-glutamyl transpeptidase
HA	Hyaluronic acid
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HH	Hereditary hemochromatosis
MMP-2	Matrix metalloproteinase-2
NAFLD	Nonalcoholic fatty liver disease
NPV	Negative predictive value
PPV	Positive predictive value
SLFG	Shanghai Liver Fibrosis Group's index
TIMP-1	Tissue inhibitor of matrix metalloproteinase-1

Key Facts of Accuracy Assessments for Biomarkers

- Area under ROC curves (AUC) is the most commonly used measurement to evaluate the accuracy of biomarkers.
- AUC value ranges from 0 to 1. Higher AUC value suggests better accuracy of the tested biomarker.
- It is generally accepted that biomarkers with AUC of 0.75 or more have acceptable accuracy and can be potentially used in clinical settings.

- Sensitivity measures the proportion of positives (i.e., have significant fibrosis) that are correctly identified by the biomarker.
- Specificity measures the proportion of negatives (i.e., do not have significant fibrosis) that are correctly identified by the biomarker.
- Positive predictive value (PPV) is the probability that subjects with a positive biomarker result are truly positive (i.e., have significant fibrosis).
- Negative predictive value (NPV) is the probability that subjects with a negative biomarker result are truly negative (i.e., do not have significant fibrosis).

Key Facts for Direct Fibrosis Biomarkers in Liver Disease

- Direct fibrosis biomarkers are those serum markers that are directly involved in the process of liver fibrosis progression.
- Direct fibrosis biomarkers are not routinely tested in clinical practice, and they have better predictive accuracy for fibrosis than routine serum tests such as ALT, AST, and platelet count.
- Commonly used direct fibrosis biomarkers can be broadly divided into three categories: glycoproteins, collagen fragments, and collagenases and their inhibitors.
- Glycoproteins include: HA, laminin, and YKL-40.
- Collagens fragments include: procollagen III N-peptide, type I collagen, type III collagen, type IV collagen, and type VI collagen.
- Collagenases and their inhibitors include: matrix metalloproteases and tissue inhibitor of metalloproteinases.

Definition of Words and Terms

Cholestasis	The obstruction of bile flow from liver to duodenum. It leads to significant serum bilirubin increase and jaundice.
Cirrhosis	The most advanced stage of liver fibrosis and is characterized by the distortion of the liver parenchyma associated with nodule formation, altered blood flow, and increased risk of liver complications and death.
Cystic fibrosis	It is an inherited multisystem disease leading to thickened mucous secretions that affects the lung, digestive system, sweat glands, and reproductive tract.
Extracellular matrix	A tightly organized molecular network secreted by cells that provides functional and structural support for liver parenchyma.

Gilberts syndrome	Also known as constitutional hepatic dysfunction or familial nonhemolytic jaundice, it is an inherited disorder of bilirubin glucuronidation. It leads to episodes of increase in unconjugated bilirubin.
Hemolysis	A condition where circulating red blood cells become fragmented and have shortened survival.
Liver decompensation	Liver decompensation occurs when clinically evident liver complication develops, including: ascites, variceal bleeding, encephalopathy, hepatorenal syndrome, spontaneous bacterial peritonitis, hepatic hydrothorax, hepatopulmonary syndrome, and non-obstructive jaundice.

Introduction

Chronic liver disease is a common health problem worldwide with complications of cirrhosis estimated to be responsible for over 1.2 million deaths globally in 2013 (Evaluation 2014). Chronic liver diseases include but are not limited to chronic hepatitis C, chronic hepatitis B, alcoholic liver disease, and nonalcoholic fatty liver disease (NAFLD). The major consequences of chronic liver disease are the development of liver decompensation or hepatocellular carcinoma (HCC), and these eventually lead to the requirement for liver transplantation or death. Most chronic liver diseases have a similar clinical course with a prolonged asymptomatic early phase during which liver damage progresses silently and a variable late clinical presentation of decompensated cirrhosis in a relative minority of patients. It has long been a challenge to identify patients with chronic liver disease who have more severe disease and have a greater risk of developing liver-related morbidity and mortality.

In patients with chronic liver disease, liver fibrosis occurs in response to chronic liver injury and results in the change in the amount, distribution, and quality of extracellular matrix in the liver (Hernandez-Gea and Friedman 2011). The disease prognosis is closely associated with the severity of liver fibrosis, and the majority of adverse outcomes occur following the development of severe liver fibrosis known as liver cirrhosis (Seeff 1997; Huang et al. 2015). Thus, liver fibrosis severity is currently the most reliable measure of patient prognosis. Consequently, the measurement of liver fibrosis may guide important clinical decisions including the need for treatment of chronic liver disease, the initiation, and the frequency of HCC and liver decompensation surveillance.

Histopathological staging of liver biopsy is traditionally acknowledged as the gold standard for liver fibrosis measurement. Metavir staging system (F0–F4) and Ishak staging system (Ishak 0–6) are the most widely used histopathological staging systems of liver fibrosis; these scoring systems stage fibrosis in a semiquantitative fashion from zero (no fibrosis) to four (Metavir) or six (Ishak) which equates to

cirrhosis. The application of liver biopsy is limited, however, due to its invasive nature, sampling error, and risk of serious complications which may (rarely) result in death (Rockey and Bissell 2006). Furthermore, liver biopsy is inconvenient, expensive, and not widely accessible to a large number of patients or physicians. In current clinical practice, it is infrequently used as a clinical prognostic tool. Surrogate biomarkers that can accurately predict the severity of liver fibrosis are of great clinical significance. Compared to liver biopsy, biomarkers have advantages of low cost, wide availability, high reproducibility, and noninvasive nature. Furthermore, they provide information on a continuous scale as opposed to the semiquantitative histological staging systems utilized in liver biopsies. Thus, noninvasive tests may be more sensitive to subtle alterations in fibrosis than biopsy and provide greater prognostic information.

Hepascore

Given the inherent limitations of liver biopsy, Hepascore was developed as a noninvasive serum model that predicted the severity of liver fibrosis in patients with chronic liver disease with the formula being $= y/(1+y)$; $y = \exp[-4.185818 - 0.0249\times \text{age} + 0.7464 \times \text{sex}(\text{male} = 1,\ \text{female} = 0) + 1.0039\times \text{alpha2-macroglobulin}(g/L) + 0.0302 \times \text{hyaluronic acid}(g/L) + 0.0691 \times \text{bilirubin}(\text{mol}/L) - 0.0012 \times \text{gamma-glutamyl transpeptidase}(U/L)]$ (Adams et al. 2005a). The Hepascore result ranges from 0 to 1. Higher values are predictive of more severe liver fibrosis whereas lower values are indicative of the absence of significant hepatic fibrosis. After the initial development and validation of Hepascore in chronic hepatitis C, Hepascore has been further validated in chronic hepatitis B, alcoholic liver disease, and NAFLD and has been demonstrated to have excellent accuracy for the detection of significant liver fibrosis (Metavir F2–F4) and cirrhosis (Metavir F4) (Becker et al. 2009; Raftopoulos et al. 2012; Naveau et al. 2009; Adams et al. 2011). It now has been widely utilized for patients with chronic liver disease in Australia, the United States, Europe, and Asia.

Four serum biomarkers are included in the Hepascore model and provide useful information to predict the severity of liver fibrosis. Hyaluronic acid (HA) is a glycosaminoglycan, which directly reflects the extracellular matrix turnover during the progression of liver fibrosis. Both the increase of HA production by activated hepatic stellate cells and decrease of HA degradation by sinusoidal endothelial cells contributed to serum HA increase in patients with liver fibrosis (McGary et al. 1989; Pares et al. 1996). Alpha2-macroglobulin is a potent protease inhibitor that is able to inactivate a variety of proteinases involved in fibrogenesis and fibrinolysis (Tiggelman et al. 1997; Ho et al. 2010). The increased expression of alpha2-macroglobulin by activated hepatic stellate cell may play an important role in the matrix remodeling in fibrotic liver (Kawser et al. 1998). Gamma-glutamyl transpeptidase (GGT) and bilirubin are included in the routine liver

function testing as they increase in diseases of the liver and biliary tract. GGT is a liver enzyme involved in hepatic glutathione metabolism and amino acid metabolism, with numerous studies demonstrating a close correlation with liver fibrosis in patients with chronic liver disease (Lemoine et al. 2016; Imbert-Bismut et al. 2001). Serum bilirubin increases in patients with liver fibrosis due to impaired hepatic excretory function and less enterohepatic circulation attributable to portal systemic shunting (Azer et al. 1993). Age and gender influence both the severity of liver fibrosis and serum biomarker levels and thus are integral parts of the model.

Hepascore in Chronic Hepatitis C

The Development of Hepascore

Hepascore was developed in 2005 using a multicenter Australian cohort of chronic hepatitis C patients (Adams et al. 2005a). In the initial study of Hepascore development, 211 untreated patients were randomized into a training set ($n = 117$) and a validation set ($n = 104$). Liver biopsy was performed at the time of serum collection, and Metavir stage was used as the reference standard. A wide range of liver fibrosis severity was observed. Forty-four percent of the included patients had significant fibrosis (Metavir F2–F4) and 6% of them had cirrhosis (Metavir F4). Ten candidate biomarkers were included in the study, namely, aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, GGT, hyaluronic acid, tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), matrix metalloproteinase-2 (MMP-2), alpha-2 macroglobulin, apolipoprotein-A1, and haptoglobin. Multivariate logistic regression was used to develop multiple predictive models, from which the model providing the greatest accuracy (determined by area under the receiver operator characteristic curve) was chosen for validation. HA, GGT, bilirubin, and alpha2-macroglobulin together with age and sex were chosen in the final model.

Hepascore significantly increased with each increase in Metavir stage (Fig. 1). It achieved excellent accuracy to predict significant fibrosis, advanced fibrosis, and cirrhosis with area under ROC curves (AUC) of 0.85, 0.96, and 0.94, respectively. Using a cut point of 0.5 enabled the detection of significant fibrosis with a sensitivity of 67% and a specificity of 92%. Applying the same cut points to detect advanced fibrosis, it achieved a sensitivity of 95% and specificity of 81%. A cut point of 0.84 was applied to detect cirrhosis with a sensitivity of 71% and a specificity of 84% (Table 1). Validation of the Hepascore model in the second set of 104 patients demonstrated similarly accurate results.

Significant fibrosis and cirrhosis are two critical fibrosis stages in clinical practice for chronic hepatitis C. The presence of significant fibrosis suggests an increased risk of developing liver-related morbidity, and thus antiviral treatment is recommended at this disease stage (EASL 2011; Omata et al. 2012; Ghany et al. 2009). Therefore, the cut point of Hepascore of 0.5 can be used to guide treatment decisions instead of

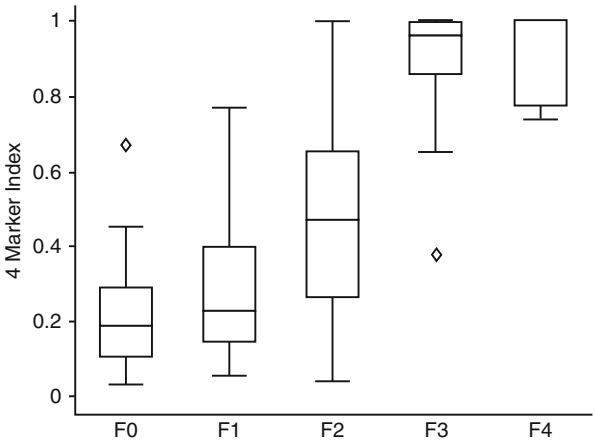

Fig. 1 Hepascore values in patients with chronic hepatitis C infection according to Metavir fibrosis stage. Upper and lower limits of boxes represent 25th and 75th percentile, respectively, with whiskers the 25th and 75th percentile ±1.53× interquartile range. *Dots* represent outliers (Taken from Adams et al. (2005a) with permission)

liver biopsy. The development of cirrhosis signifies a dramatically increased risk of liver decompensation, HCC development, and liver-related death and precipitates surveillance for esophageal varices and HCC (Castera 2012). Patients with Hepascore greater than 0.84 are recommended to undergo further investigation to confirm the diagnosis of cirrhosis; once confirmed, they should commence surveillance and monitoring for decompensation and HCC. On the other hand, asymptomatic patients with Hepascore less than 0.84 will have small possibility of cirrhosis development, and Hepascore tests are recommended to monitor fibrosis progression for those patients.

Validation Studies of Hepascore to Detect Liver Fibrosis in Chronic Hepatitis C

The diagnostic accuracy of Hepascore has been extensively validated in over 4,000 subjects from around the world (Table 1). The two largest validation studies were performed in France using 1,056 chronic hepatitis C patients (Cales et al. 2008; Boursier et al. 2009). These two studies demonstrated that Hepascore had excellent diagnostic accuracy for significant fibrosis, advanced fibrosis, and cirrhosis with AUC of 0.78, 0.83, and 0.90, respectively. Hepascore showed good to excellent diagnostic performance among all other validation studies although slightly different cut points have been proposed depending upon the underlying prevalence (or pretest probability) of fibrosis. A cut point of 0.32–0.34 was suggested by several studies to detect significant fibrosis. It is generally agreed by all studies to use a Hepascore of 0.5–0.6 to detect advanced fibrosis and a Hepascore of 0.80–0.84 for cirrhosis. Validations studies have confirmed that Hepascore less than 0.5 is highly predictive of the absence of advanced fibrosis with negative predictive value (NPV) of 90–92% (Boursier et al. 2009; Leroy et al. 2007). A cut point of <0.84 also achieved a high accuracy to exclude cirrhosis with NPV of

Table 1 Studies examining the accuracy of Hepascore in detecting liver fibrosis in chronic hepatitis C infection

Author	Year	Country	No.	Significant fibrosis						Advanced fibrosis						Cirrhosis					
					Cut point	Sen	Spe	PPV	NPV	AUC	Cut point	Sen	Spe	PPV	NPV	AUC	Cut point	Sen	Spe	PPV	NPV
Adams (Adams et al. 2005a)	2005	Australia	221	0.82	0.50	63%	89%	88%	–	0.90	0.50	88%	74%	–	98%	0.84	0.89	71%	89%	–	98%
Halfon (Halfon et al. 2007)	2007	France	356	0.76	0.32	77%	63%	59%	80%	0.81	0.53	78%	72%	32%	95%	0.89	0.61	92%	72%	11%	100%
Leroy (Leroy et al. 2007)	2007	France	180	0.79	0.50	54%	84%	78%	64%	0.85	0.50	77%	81%	62%	90%	–	–	–	–	–	–
Bourliere (Bourliere et al. 2008)	2008	France	467	0.82	0.50	63%	86%	82%	70%	0.84	–	–	–	–	–	0.90	0.84	71%	88%	33%	97%
Cales (Cales et al. 2008)	2008	France	1056	0.78	0.47	66%	79%	78%	68%	0.83	–	–	–	–	–	0.90	–	–	–	–	–
Boursier (Boursier et al. 2009)	2009	France	1056	–	–	–	–	–	–	0.83	0.5	82%	71%	48%	92%	0.90	0.80	80%	83%	37%	97%
Becker (Becker et al. 2009)	2009	USA	391	0.81	0.55	82%	65%	70%	78%	0.83	–	–		–	–	0.88	–	–	–	–	–
Guéchot (Guechot et al. 2010)	2010	France	512	0.81	0.50	77%	70%	71%	77%	0.82	0.60	80%	70%	54%	89%	0.88	0.75	86%	74%	37%	97%
Kalantari (Kalantari et al. 2011)	2011	Iran	80	–	0.34	67%	56%	64%	56%	–	0.61	82%	86%	70%	92%	–	0.84	100%	97%	89%	100%
Crisan (Crisan et al. 2012)	2012	Romania	446	0.69	0.34	57%	72%	82%	43%	0.71	0.61	61%	73%	50%	81%	–	–	–	–	–	–
Zarski (Zarski et al. 2012)	2012	France	382	0.82	0.50	75%	73%	70%	77%	–	–	–	–	–	–	0.89	0.84	77%	81%	41%	95%
Leroy (Leroy et al. 2014)	2014	France	255	0.77	0.34	74%	64%	69%	69%	0.86	0.47	79%	85%	53%	95%	0.88	0.64	78%	82%	34%	97%

AUC area under the curve, *Sens* sensitivity, *Spe* specificity, *PPV* positive predictive value, *NPV* negative predictive value

95–97% (Boursier et al. 2009; Zarski et al. 2012). The cut point of ≥0.5 is moderately predictive of the present of significant fibrosis with positive predictive value (PPV) of 71–78% (Cales et al. 2008; Zarski et al. 2012; Guechot et al. 2010; Leroy et al. 2007).

A number of studies have compared the diagnostic performance of Hepascore with other commonly used serum models for liver fibrosis. FibroTest, FibroMeter, and APRI are the widely validated serum models in chronic hepatitis C. FibroTest includes alpha2-macroglobulin, haptoglobin, apolipoprotein A1, GGT, and bilirubin (Imbert-Bismut et al. 2001). FibroMeter includes platelet count, α2-macroglobulin, AST, age, prothrombin index, HA, and blood urea nitrogen (Cales et al. 2005). APRI has advantage in its simplicity with only platelet count and AST level included in the model (Wai et al. 2003). Other commonly studied serum models include FIB-4 (Sterling et al. 2006), ELF (Rosenberg et al. 2004), FibroIndex (Koda et al. 2007), Forn's index (Forns et al. 2002), FIBROSpect II (Patel et al. 2004), Lok index (Lok et al. 2005), GUCI (Islam et al. 2005), and SHASTA (Kelleher et al. 2005). Although different biomarkers are present in different serum models, most models achieve similar accuracy with an AUC larger than 0.75 to predict significant fibrosis and an AUC larger than 0.85 to predict cirrhosis. A recent systemic review of studies that evaluated the predictive accuracy of biomarkers in chronic hepatitis C summarized 68 studies which performed direct comparison between two or more biomarkers (Chou and Wasson 2013). Hepascore was compared with APRI to predict significant or advanced fibrosis in six studies and compared with FibroTest in ten studies. There is no significant difference in AUC to predict significant or advanced fibrosis between Hepascore and APRI [median AUC difference −0.01 (range: −0.04 to 0.06]. Similarly, there was no difference in AUC between Hepascore and FibroTest [median AUC difference −0.02 (rang: −0.09 to 0.06)]. Three studies compared Hepascore and APRI to predict cirrhosis; Hepascore showed a nonsignificant higher predictive accuracy with the median difference in AUC of 0.03 (range: −0.3 to 0.03). Six studies compared Hepascore with FibroTest to predict cirrhosis. The median difference in AUC was 0.02 (−0.03 to 0.03). No significant difference in the diagnostic accuracy among serum models was found in most comparison studies (Chou and Wasson 2013).

Hepascore values are dynamic over time and serial measurements may be useful to monitor liver fibrosis in patients over time. A cohort study of 356 patients with chronic hepatitis C who had two Hepascore values 3.3 years apart demonstrated that those with a baseline Hepascore value >0.75 and increasing Hepascore values over time were at greater risk of developing liver-related death, HCC, or decompensation over a mean 5.5-year follow-up (Jeffrey et al. 2015). The impact of eradication of the hepatitis C infection on serial Hepascore measurements and prognostication is unknown. As the efficacy of antiviral treatment is rapidly increasing, it will become increasingly important to be able to predict patients who remain at risk of liver-related morbidity and mortality despite viral eradication. Further studies of Hepascore in this setting are required.

Hepascore in Chronic Hepatitis B

After the initial development of Hepascore in chronic hepatitis C, the utility of Hepascore was extended to other types of chronic liver disease. Up to now, there are six studies involving more than 800 patients which have examined the predictive ability of Hepascore in chronic hepatitis B infection. These studies demonstrate that Hepascore has similarly accuracy in the prediction of fibrosis in chronic hepatitis B compared to that in chronic hepatitis C (Table 2). The AUC for Hepascore ranged 0.74–0.83 to predict significant fibrosis, 0.78–0.95 to predict advanced fibrosis, and 0.78–0.92 to predict cirrhosis. A cut point of 0.32–0.50 was applied to detect significant fibrosis, a cut point of 0.42–0.76 was suggested for advanced fibrosis, and a cut point of 0.52–0.90 was chosen for cirrhosis. Hepascore had excellent accuracy to exclude advanced fibrosis and cirrhosis using a cut point of 0.72 and 0.87, respectively. A cut point of <0.72 has a NPV of 90% to exclude advance fibrosis, and a cut point of <0.87 achieved a NPV of 99% to exclude cirrhosis (Raftopoulos et al. 2012; Chen et al. 2013). A cut point of ≥ 0.50 had moderate predictive ability for the presence of significant fibrosis with a PPV of 69% (Chen et al. 2013).

The diagnostic accuracy of Hepascore in hepatitis B has been compared with other serum models developed in chronic hepatitis C cohorts as well as those specifically developed in chronic hepatitis B cohorts (Shanghai Liver Fibrosis Group's index (SLFG) (Zeng et al. 2005), S-index (Zhou et al. 2010), Hui's index (Hui et al. 2005)). A Turkish study compared Hepascore with APRI, Forn's index, FIB-4, S-index, and SLFG using 76 chronic hepatitis B patients (Basar et al. 2013). Hepascore had the greatest predictive ability compared to other serum models for significant fibrosis, advanced fibrosis, and cirrhosis with an AUC of 0.78, 0.78, and 0.82, respectively. Forn's index was the second best to predict significant fibrosis and S-index ranked the second to predict cirrhosis. APRI had the lowest predictive accuracy among serum models in this cohort. Another study compared APRI, FIB-4, Forn's index, FibroMeter, Hepascore, and SLFG in a Chinese cohort of 78 chronic hepatitis B patients (Wu et al. 2010). It concluded that those serum models including direct fibrosis biomarkers such as Hepascore, SLFG, and FibroMeter had greater predictive performance than those models without direct fibrosis biomarkers, namely: APRI, Forn's index, and FIB-4. A third study compared eleven biomarkers in a population of 108 patients with HBV/HIV coinfection (Bottero et al. 2009). Included biomarkers were FibroTest, FibroMeter, SHASTA, Hepascore, SLFG, APRI, FIB-4, Forn's index, AST/ALT ratio, HA, and Hui's index. FibroTest, FibroMeter, Hepascore, and SLFG showed the best predictive performance for liver fibrosis in HBV/HIV coinfected patients. No study to date has evaluated the ability of Hepascore to predict adverse clinical outcomes for chronic hepatitis B patients.

In contrast to chronic hepatitis C, factors in addition to fibrosis stage, such as hepatitis B viral load and ALT levels, are important to guide patient prognosis and treatment decisions in chronic hepatitis B. Liver biopsy and antiviral treatment are recommended by current clinical guidelines for those patients with abnormal ALT levels or those with significant fibrosis (Sorrell et al. 2009). Notably, a normal ALT level does not exclude significant fibrosis or even cirrhosis (Kumar et al. 2008; Lai

Table 2 Studies examining the accuracy of Hepascore in detecting liver fibrosis in chronic hepatitis B infection

				Significant fibrosis						Advanced fibrosis						Cirrhosis					
Author	Year	Country	No.	AUC	Cut point	Sen	Spe	PPV	NPV	AUC	Cut point	Sen	Spe	PPV	NPV	AUC	Cut point	Sen	Spe	PPV	NPV
Bottero (Bottero et al. 2009)	2009	France	108[a]	0.74	0.48	67%	68%	77%	57%	0.83	0.76	72%	87%	76%	84%	0.92	0.90	80%	89%	60%	96%
Zhou (Zhou et al. 2010)	2010	China	146	0.77	0.50	–	–	–	–	0.82	–	–	–	–	–	0.78	–	–	–	–	–
Wu (Wu et al. 2010)	2010	China	78	0.80	0.50	88%	50%	55%	85%	0.95	–	–	–	–	–	–	–	–	–	–	–
Raftopoulos (Raftopoulos et al. 2012)	2011	Australia	179	0.83	0.50	79%	74%	69%	83%	0.87	–	–	–	–	–	0.91	0.88	87%	86%	36%	99%
Basar (Basar et al. 2013)	2013	Turkey	76	0.78	0.32	78%	68%	83%	61%	0.78	0.50	71%	79%	40%	83%	0.82	0.52	85%	75%	41%	96%
Leroy (Leroy et al. 2014)	2014	France	255	0.75	0.32	71%	69%	69%	71%	0.82	0.42	75%	71%	95%	90%	0.86	0.55	72%	84%	97%	96%

[a]HBV/HIV coinfected patients. *AUC* area under the curve, *Sens* sensitivity, *Spe* specificity, *PPV* positive predictive value, *NPV* negative predictive value

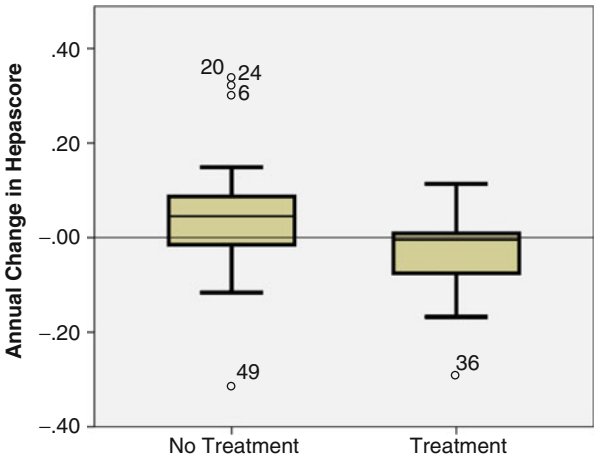

Fig. 2 Annual rate of change in serum Hepascore values in patients with chronic hepatitis B infection with or without antiviral treatment. Hepascore increased significantly in patients with chronic hepatitis B infection who were not being treated with likely progressive fibrosis, whereas Hepascore values decreased significantly among individuals receiving antiviral treatment associated with fibrosis regression (Taken from Raftopoulos et al. (2012) with permission)

et al. 2007). Nevertheless, patients with normal ALT levels are often just monitored without antiviral treatment; however, subjects with occult fibrosis may be inappropriately not referred for treatment if fibrosis is not evaluated. One study evaluated the predictive accuracy of Hepascore among 73 patients with normal or near-normal ALT levels (<60 IU/L) (Raftopoulos et al. 2012); 26% of the included patients had significant fibrosis on biopsy, 15.1% of them had advanced fibrosis, and 2.7% had cirrhosis. Among these patients with low/normal serum ALT levels, Hepascore remained accurate for the prediction of significant fibrosis, advanced fibrosis, and cirrhosis with AUC of 0.79, 0.85, and 0.80, respectively. Thus Hepascore may be used among those patients with normal or near-normal ALT levels to detect occult fibrosis and therefore determine the need for antiviral therapy.

Hepascore may also be useful for monitoring hepatic fibrosis over time and in response to antiviral therapy. Hepatitis B treatment reverses fibrosis, whereas the natural history of untreated active CHB is progressive fibrosis. Correspondingly, in a small cohort of 40 patients followed for up to 8.7 years, Hepascore values increased in the absence of treatment but fell during therapy (Fig. 2). This suggests Hepascore is an accurate and dynamic noninvasive marker of fibrosis in hepatitis B infection.

Hepascore in NAFLD

NAFLD is the most common chronic liver disease in Western countries where it affects approximately 20–30% of the population. Compared to viral hepatitis, NAFLD has relatively benign disease course. The majority of NAFLD patients

will not develop liver-related morbidity and mortality in their lifetime, as only 5% of NAFLD patients progress to cirrhosis and 1.7% of patients die from liver disease over a decade (Adams et al. 2005b). The management of NAFLD patients focuses upon implementing lifestyle modifications, such as weight loss through dietary changes and exercise and reduced alcohol consumption (GESA 2013). However, current guidelines recommend those with significant fibrosis should be considered for liver-specific pharmacotherapy such as vitamin E or pioglitazone or should be offered a clinical trial. Additionally, a small portion of NAFLD patients with cirrhosis will require further management such as surveillance for esophageal varices and HCC. Hepascore has the potential to be used as a screening method for the selection of those patients with more severe liver fibrosis and higher risk of liver-related morbidity and mortality.

One study has validated the accuracy of Hepascore to predict liver fibrosis in NAFLD patients (Adams et al. 2011). Two hundred forty-two NAFLD patients were included in this study: 40.1% of the included patients had significant fibrosis, 21.9% had advanced fibrosis, and 9.3% had cirrhosis. Hepascore achieved the AUC of 0.73, 0.81, and 0.91 to detect significant fibrosis, advanced fibrosis, and cirrhosis, respectively. A cut point of 0.44 achieved a sensitivity of 51%, specificity of 88%, PPV of 74%, and NPV of 73% to detect significant fibrosis. A cut point of 0.37 had a sensitivity of 76%, specificity of 84%, PPV of 57%, and NPV of 92% for advanced fibrosis. A cut point of 0.70 was chosen to predict cirrhosis with a sensitivity of 87%, specificity of 89%, PPV of 45%, and NPV of 99%. Importantly, a cut point of <0.61 achieved excellent accuracy to exclude advanced fibrosis and cirrhosis with NPV of 90%. This cut point can be used to screen for those patients who have more severe liver fibrosis and thus require further management.

This study also compared the predictive accuracy of Hepascore with other serum models including: FIB-4, FibroTest, APRI, and BARD index (Fig. 3). BARD index was specifically developed in cohort of NAFLD patients and includes the covariates of body mass index, AST, ALT, and diabetes (Harrison et al. 2008). Results demonstrated that serum models that included direct fibrosis biomarkers (Hepascore, FibroTest, FIB-4) had better predictive performance than those serum models that only include variables from routine clinical assessment (APRI and BARD).

Hepascore in Alcoholic Liver Disease

Excessive alcohol consumption can result in the development of alcoholic fatty liver disease, alcoholic hepatitis, and alcoholic cirrhosis. The diagnosis of alcoholic liver disease is based on the following features: history of alcohol abuse, clinical evidence of liver disease, and the results of laboratory tests. Nevertheless, a minority of individuals who consistently drink at harmful levels will develop liver cirrhosis. Thus it is necessary to prognosticate risk according to the degree of underlying liver fibrosis.

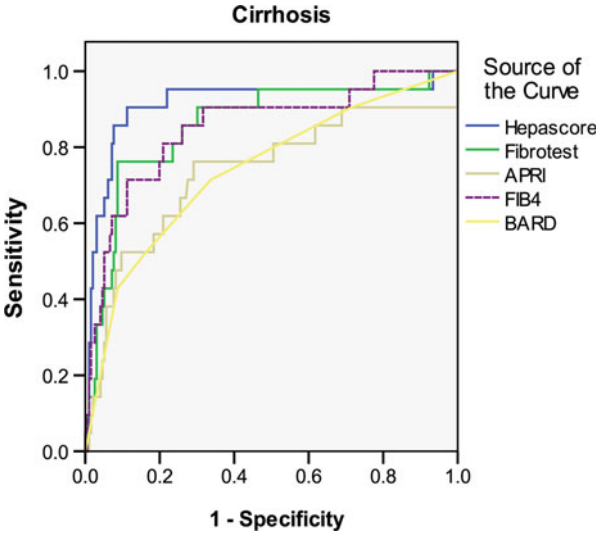

Fig. 3 Receiver operator characteristic (*ROC*) curve of noninvasive serum markers for the prediction of cirrhosis in nonalcoholic fatty liver disease. Hepascore had the highest accuracy as determined by the area under the ROC curve (Taken from Adams et al. (2011) with permission)

Hepascore has been examined in French cohorts of subjects with alcoholic liver disease and compared to other noninvasive models. One study evaluated the predictive ability of Hepascore, FibroTest, FibroMeter, APRI, PGA, PGAA, and hyaluronic acid tests in 103 patients with alcoholic liver disease (Nguyen-Khac et al. 2008). PGA index was developed in alcoholic liver disease and includes prothrombin time, gamma-glutamyl transpeptidase, and apolipoprotein AI (Poynard et al. 1991). PGAA index was the modified model of PGA index with an additional biomarker alpha2-macroglobulin and achieved improved predictive accuracy for liver fibrosis in alcoholic liver disease (Naveau et al. 1994). This study found Hepascore had AUC of 0.76, 0.83, and 0.76 to predict significant fibrosis, advanced fibrosis, and cirrhosis, respectively. The predictive accuracy of Hepascore was similar with APRI and HA, but was lower than FibroTest, FibroMeter, and PGAA.

A larger study evaluated the predictive performance of six serum models (Hepascore, FibroMeter, FibroTest, APRI, Forn's index, and FIB-4) in 218 patients with alcoholic liver disease (Naveau et al. 2009). Hepascore achieved an AUC of 0.83 and 0.92 to predict significant fibrosis and cirrhosis, respectively. Hepascore was able to successfully classify 60% of patients for significant fibrosis by using two cut points: 0.25 and 0.94. The cut point of 0.25 achieved a sensitivity of 90% and the cut point of 0.94 had a specificity of 90%. For the prediction of cirrhosis, a cut point of 0.97 achieved a sensitivity of 90%, and a cut point of 0.99 achieved a specificity of 90%. Ninety-four percent of patients could be successfully classified using these two cut points. The predictive abilities of Hepascore were similar with FibroMeter and FibroTest for significant fibrosis and cirrhosis but were significantly greater than APRI, Forn's index, and FIB-4.

Hepascore in Other Chronic Liver Diseases

Hereditary hemochromatosis (HH) is a relatively rare disorder that can lead to chronic liver injury and liver fibrosis. A C282Y mutation in the hemochromatosis gene (*HFE*) is the principal cause of HH and leads to systemic iron overload and end-organ damage in the liver, heart, and endocrine organs. An American study screened 16,000 individuals and found that 0.4% population were C282Y homozygotes (Pankow et al. 2008). Hepascore values were significantly higher in C282Y homozygotes compared to age-matched wild-type individuals (Pankow et al. 2008). Another French study performed Hepascore on 57 HH patients (Adhoute et al. 2008). Liver biopsy was not performed for participants. The mean Hepascore was 0.39 for HH patients, and a significant correlation was found between Hepascore and liver stiffness, which is another noninvasive measure of hepatic fibrosis. Hepascore will also be used to assess treatment response for HH patients in an Australian clinical trial (Ong et al. 2015).

Thalassemia is characterized by reduced or absent production of one of the globin chains of hemoglobin. Patients with transfusion-dependent thalassemia major often develop liver fibrosis due to liver iron overload and/or hepatitis C virus (HCV) infection. A study performed Hepascore test in 201 patients with transfusion-dependent thalassemia major (Papastamataki et al. 2010). The mean Hepascore was 0.86 in this cohort and 55% of them had a Hepascore $\geq$0.42 (Papastamataki et al. 2010). This study confirms the applicability of Hepascore in a population at risk of liver disease and in whom liver biopsy is contraindicated due to excessive bleeding risk.

Cystic fibrosis may cause liver fibrosis and portal hypertension with subsequent risk of complications such as ascites and variceal bleeding. A study of 25 patients with cystic fibrosis liver disease (CFLD) and 25 patients who had cystic fibrosis without liver disease (Kitson et al. 2013) found Hepascore to be significantly higher in those patients with liver disease compared to those without (mean: 0.45 vs 0.31). Hepascore had an AUC of 0.69 to detect CFLD and an AUC of 0.85 to predict portal hypertension in cystic fibrosis patients. A cut point of 0.48 achieved a sensitivity of 88% and a specificity of 83% to detect portal hypertension. The same cut point achieved a sensitivity of 88%, specificity of 82%, PPV of 70%, and NPV of 93% to predict portal hypertension in those patients with CFLD.

Hepascore has also been examined in the setting of monitoring for methotrexate hepatotoxicity. One small study of 24 psoriasis patients found that Hepascore was correlated with the severity of psoriasis, although liver biopsy to assess severity of fibrosis was not performed (Chladek et al. 2013). HA localizes in the dermis, epidermis, and stratum corneum and is upregulated in psoriasis (Wells et al. 1991). Thus, active psoriasis may result in a falsely elevated score; however, further studies are required to confirm this.

Liver transplantation is an important treatment option for patients with end-stage liver disease and primary hepatic malignancy. Nevertheless, liver

disease frequently recurs posttransplantation, and the rate of liver fibrosis progression post-liver transplantation is significantly higher than that of pre-liver transplantation (Berenguer et al. 2000). Thus fibrosis evaluation is particularly important in this group of patients where death from recurrent liver disease is a leading cause of mortality. A study of 231 liver transplantation patients evaluated the predictive accuracy of noninvasive serum models (Beckebaum et al. 2010). Seventy-eight patients were transplanted for HCV-related cirrhosis, and 153 were transplanted for non-HCV diseases. Hepascore had an AUC of 0.80 to predict advanced fibrosis in HCV patients with a cut point of 0.83 achieving a sensitivity of 52%, specificity of 92%, PPV of 81%, and NPV of 65%. The AUC for Hepascore to predict advanced fibrosis in non-HCV patients was 0.77 with a cut point of 0.66 achieving a sensitivity of 43%, specificity of 91%, PPV of 65%, and NPV of 80%. The AUC of Hepascore to predict cirrhosis was 0.94 for HCV patients and 0.85 for non-HCV patients. This study also compared the predictive ability of Hepascore with other serum models (FibroTest, FibroIndex, Lok index, FIB-4, APRI, and Forn's index). Hepascore had comparable or greater accuracy compared to the other serum models, highlighting its utility in this difficult population.

Potential Applications to Prognosis, Other Diseases, or Conditions

Liver fibrosis is a marker of disease severity in patients with chronic liver disease. Correspondingly, fibrosis is prognostic for the development of disease-related morbidity which manifests as hepatic decompensation (with the development of ascites, variceal bleeding, hepatic encephalopathy, or hepatorenal syndrome), HCC, and liver-related death or liver transplantation (Huang et al. 2015). Although noninvasive fibrosis markers such as Hepascore have been developed to predict fibrosis, their validity as prognostic markers is dependent upon their ability to predict disease outcomes. Studies examining the prognostic ability of noninvasive liver fibrosis markers are limited due to the large number of subjects and long duration of follow-up that is required. Nevertheless, the prognostic accuracy of Hepascore has been confirmed in three cohort studies of patients with chronic hepatitis C infection and alcoholic liver disease (Chinnaratha et al. 2014; Boursier et al. 2014; Naveau et al. 2009).

Two cohort studies of patients with chronic hepatitis C have demonstrated that Hepascore is accurate at predicting liver-related decompensation and death. Firstly, a study of 406 patients followed for a mean of 6 years found at the end of follow-up, 16 patients had developed liver decompensation, 4 patients had been diagnosed with HCC, and 14 patients had died from their liver disease or had undergone liver transplantation. Patients with a baseline Hepascore $\geq$0.5 had 33 (95% confidence interval (CI) 4–250) times higher risk of dying from their liver disease and had 12 (95% CI 3–41) times higher risk of developing liver decompensation or HCC than those with Hepascore $<$0.5. When Hepascore values were analyzed in quartiles,

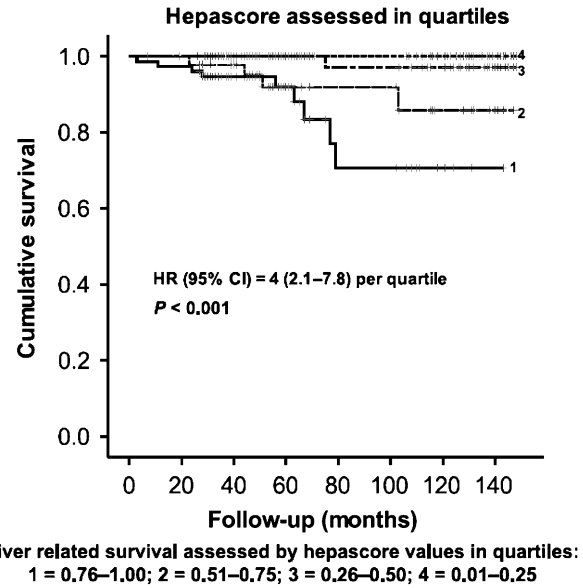

Fig. 4 Prediction of liver-related survival according to baseline Hepascore values in 406 subjects with chronic hepatitis C infection. The risk of liver-related death or transplantation increases with increasing Hepascore quartile (Taken from Chinnaratha et al. (2014) with permission)

liver-related mortality occurred in 11.8% of those patients with Hepascore $\geq$0.75, 8.7% of those with Hepascore of 0.5–0.75, and 0.9% of those with Hepascore of 0.25–0.5. No liver-related mortality was observed in patients who had a Hepascore value less than 0.25 over the follow-up period (Fig. 4). This study also compared the predictive ability of Hepascore with liver biopsy and other serum models (FIB-4 and APRI). Hepascore had a similar prognostic accuracy with liver biopsy, while FIB-4 and APRI were not predictive of clinical outcomes in this cohort. A second study of 373 French chronic hepatitis C patients followed for a median of 9.5 years found Hepascore was accurate at predicting the first significant liver-related decompensation and liver-related death with AUC values of 0.81 (95% CI 0.73–0.83) and 0.89 (95% CI 0.81–0.95), respectively (Boursier et al. 2014). Notably, Hepascore was as accurate as liver biopsy for predicting these outcomes.

Hepascore has also been validated as a prognostic marker of outcomes in alcoholic liver disease (Naveau et al. 2009). Among 218 patients followed for a median of 8.2 years, 85 deaths occurred including 42 liver-related deaths. Hepascore achieved an AUC of 0.78 to predict liver-related death and an AUC of 0.69 for overall survival. The prognostic performance Hepascore was similar with liver biopsy, FibroTest, and FibroMeter and was significantly greater than that of APRI, Forn's index, and FIB-4.

These studies confirm the validity of Hepascore as a prognostic marker in chronic hepatitis C infection and alcoholic liver disease. Subjects with chronic hepatitis C and low Hepascore values (<0.5) are at very low risk of liver morbidity or mortality in the medium term (5–10 years) and can be monitored if treatment is deferred. Confirmatory studies in other etiologies of liver disease including NAFLD are eagerly waited.

Hepascore Advantages and Limitations

Hepascore is simple, noninvasive, cheap, accurate, extensively validated, and widely accessible. The primary utility of Hepascore is to detect the severity of liver fibrosis in chronic liver disease. Moreover, Hepascore can be further used to monitor liver fibrosis progression or regression, to assess treatment efficacy, and to predict clinical outcomes.

Hepascore has several limitations. Firstly, there are several conditions other than liver fibrosis that can cause the increase of Hepascore and lead to false positive of Hepascore results. Inflammatory states may increase HA and alpha-2 macroglobulin levels as acute phase reactants. Acute hepatitis or drugs which induce GGT levels may lead to falsely high readings. Similarly, cholestasis, Gilbert's syndrome, and hemolysis may increase bilirubin levels in the absence of hepatic fibrosis, and interpretation of Hepascore results in these states should be done with caution. Importantly, Hepascore should not be interpreted as a stand-alone test of liver fibrosis and should always be interpreted in conjunction with other clinical, laboratory, and imaging findings.

Secondly, biological variation may cause significant within-individual variability in Hepascore results. One study collected four serial serum samples at weekly intervals from healthy volunteers and patients with chronic hepatitis B, chronic hepatitis C, and NAFLD and calculated the within-individual variations for HA, alpha2-macroglobulin, and Hepascore in each group (Rossi et al. 2013). Hyaluronic acid displayed large within-individual variation; the coefficients of variation (CV) values were 62% in healthy subjects, 38% in hepatitis C, 37% in hepatitis B, and 36% in NAFLD patients. This may in part be due to differences in the fasting status between assessments, as HA increases significantly postprandially (Khoo et al. 2012). Alpha2-macroglobulin was much less variable with CV ranging from 4.4% to 7.6% among groups. Hepascore had a reduced variability compared to that of HA, with a CV of 43% in healthy subjects, 24% in hepatitis C, 28% in hepatitis B, and 39% in NAFLD patients. Thus, Hepascore assessments should be performed in a standardized fashion in a fasting state.

Lastly, similar to other serum models, there is a significant overlap of Hepascore values between individual fibrosis stages, and these led to unacceptable accuracy of Hepascore to predict intermediate fibrosis stages (e.g., Metavir 1–2). Consequently, serum tests are limited to the prediction of binary outcomes such as significant fibrosis, advanced fibrosis, and cirrhosis, respectively. Notably, scores toward the ends of the spectrum of results tend to have greater predictive values for either the presence of absence of advanced fibrosis.

Conclusion

Hepascore is a serum-based test that quantifies hepatic fibrosis in chronic liver disease and is composed of readily available biochemical analytes in conjunction with age and sex. Hepascore is marketed across the USA by Quest Diagnostics

(New Jersey, USA), is a government rebated item in France and is recommended by the French National Authority for Health as a routine diagnostic test in chronic hepatitis C, and has now been incorporated into hepatology practices around the world. It has been validated as a marker to predict clinical outcomes and death in chronic hepatitis C and alcoholic liver disease, confirming its utility as a prognostic tool. Awareness of its limitations is important in test interpretation; however, it remains a useful clinical tool for the evaluation of patients with chronic liver disease.

Summary Points

- Hepascore is a serum model that was initially developed to predict the severity of liver fibrosis in patients with chronic hepatitis C infection.
- Hepascore model includes: HA, GGT, alpha2-macroglobulin, bilirubin, age, and sex.
- Following initial development, Hepascore has been validated worldwide in chronic hepatitis C, chronic hepatitis B, NAFLD, alcoholic liver disease, and other types of chronic liver disorders.
- Hepascore has moderate to excellent predictive ability for significant fibrosis, advanced fibrosis, and cirrhosis in chronic liver disease.
- Compared to liver biopsy, Hepascore has its advantages in its low cost, wide availability, reproducibility, and noninvasive nature.
- The limitations of Hepascore include: potential for false positives, within-individual variability, and limited predictive accuracy for lower degrees of liver fibrosis.

References

Adams LA, Bulsara M, Rossi E, Deboer B, Speers D, George J, Kench J, Farrell G, Mccaughan GW, Jeffrey GP. Hepascore: an accurate validated predictor of liver fibrosis in chronic hepatitis C infection. Clin Chem. 2005a;51:1867–73.

Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, Angulo P. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology. 2005b;129:113–21.

Adams LA, George J, Bugianesi E, Rossi E, De Boer WB, Van Der Poorten D, Ching HL, Bulsara M, Jeffrey GP. Complex non-invasive fibrosis models are more accurate than simple models in non-alcoholic fatty liver disease. J Gastroenterol Hepatol. 2011;26:1536–43.

Adhoute X, Foucher J, Laharie D, Terrebonne E, Vergniol J, Castera L, Lovato B, Chanteloup E, Merrouche W, Couzigou P, De Ledinghen V. Diagnosis of liver fibrosis using FibroScan and other noninvasive methods in patients with hemochromatosis: a prospective study. Gastroenterol Clin Biol. 2008;32:180–7.

Azer SA, Murray M, Farrell GC, Stacey NH. Selectivity and sensitivity of changes in serum bile acids during induction of cirrhosis in rats. Hepatology. 1993;18:1224–31.

Basar O, Yimaz B, Ekiz F, Ginis Z, Altinbas A, Aktas B, Tuna Y, Coban S, Delibas N, Yuksel O. Non-invasive tests in prediction of liver fibrosis in chronic hepatitis B and comparison with post-antiviral treatment results. Clin Res Hepatol Gastroenterol. 2013;37:152–8.

Beckebaum S, Iacob S, Klein CG, Dechene A, Varghese J, Baba HA, Sotiropoulos GC, Paul A, Gerken G, Cicinnati VR. Assessment of allograft fibrosis by transient elastography and noninvasive biomarker scoring systems in liver transplant patients. Transplantation. 2010;89:983–93.

Becker L, Salameh W, Sferruzza A, Zhang K, Ng Chen R, Malik R, Reitz R, Nasser I, Afdhal NH. Validation of hepascore, compared with simple indices of fibrosis, in patients with chronic hepatitis C virus infection in United States. Clin Gastroenterol Hepatol. 2009;7:696–701.

Berenguer M, Ferrell L, Watson J, Prieto M, Kim M, Rayon M, Cordoba J, Herola A, Ascher N, Mir J, Berenguer J, Wright TL. HCV-related fibrosis progression following liver transplantation: increase in recent years. J Hepatol. 2000;32:673–84.

Bottero J, Lacombe K, Guechot J, Serfaty L, Miailhes P, Bonnard P, Wendum D, Molina JM, Lascoux-Combe C, Girard PM. Performance of 11 biomarkers for liver fibrosis assessment in HIV/HBV co-infected patients. J Hepatol. 2009;50:1074–83.

Bourliere M, Penaranda G, Ouzan D, Renou C, Botta-Fridlund D, Tran A, Rosenthal E, Wartelle-Bladou C, Delasalle P, Oules V, Portal I, Castellani P, Lecomte L, Rosenthal-Allieri MA, Halfon P. Optimized stepwise combination algorithms of non-invasive liver fibrosis scores including Hepascore in hepatitis C virus patients. Aliment Pharmacol Ther. 2008;28:458–67.

Boursier J, Bacq Y, Halfon P, Leroy V, De Ledinghen V, De Muret A, Bourliere M, Sturm N, Foucher J, Oberti F, Rousselet MC, Cales P. Improved diagnostic accuracy of blood tests for severe fibrosis and cirrhosis in chronic hepatitis C. Eur J Gastroenterol Hepatol. 2009;21:28–38.

Boursier J, Brochard C, Bertrais S, Michalak S, Gallois Y, Fouchard-Hubert I, Oberti F, Rousselet MC, Cales P. Combination of blood tests for significant fibrosis and cirrhosis improves the assessment of liver-prognosis in chronic hepatitis C. Aliment Pharmacol Ther. 2014;40:178–88.

Cales P, Oberti F, Michalak S, Hubert-Fouchard I, Rousselet MC, Konate A, Gallois Y, Ternisien C, Chevailler A, Lunel F. A novel panel of blood markers to assess the degree of liver fibrosis. Hepatology. 2005;42:1373–81.

Cales P, De Ledinghen V, Halfon P, Bacq Y, Leroy V, Boursier J, Foucher J, Bourliere M, De Muret A, Sturm N, Hunault G, Oberti F. Evaluating the accuracy and increasing the reliable diagnosis rate of blood tests for liver fibrosis in chronic hepatitis C. Liver Int. 2008;28:1352–62.

Castera L. Noninvasive methods to assess liver disease in patients with hepatitis B or C. Gastroenterology. 2012;142:1293–302.

Chen YP, Peng J, Hou JL. Non-invasive assessment of liver fibrosis in patients with chronic hepatitis B. Hepatol Int. 2013;7:356–68.

Chinnaratha MA, Jeffrey GP, Macquillan G, Rossi E, De Boer BW, Speers DJ, Adams LA. Prediction of morbidity and mortality in patients with chronic hepatitis C by non-invasive liver fibrosis models. Liver Int. 2014;34:720–7.

Chladek J, Simkova M, Vaneckova J, Hroch M, Vavrova J, Hulek P. Assessment of methotrexate hepatotoxicity in psoriasis patients: a prospective evaluation of four serum fibrosis markers. J Eur Acad Dermatol Venereol. 2013;27:1007–14.

Chou R, Wasson N. Blood tests to diagnose fibrosis or cirrhosis in patients with chronic hepatitis C virus infection: a systematic review. Ann Intern Med. 2013;158:807–20.

Crisan D, Radu C, Lupsor M, Sparchez Z, Grigorescu MD, Grigorescu M. Two or more synchronous combination of noninvasive tests to increase accuracy of liver fibrosis assessment in chronic hepatitis C; results from a cohort of 446 patients. Hepat Mon. 2012;12:177–84.

EASL. EASL clinical practice guidelines: management of hepatitis C virus infection. J Hepatol. 2011;55:245–64.

Evaluation I F H M A 2014. Age-sex specific all-cause and cause-specific mortality 1990–2013. Global Burden of Disease Study 2013. Seattle: Institute for Health Metrics and Evaluation.

Forns X, Ampurdanes S, Llovet JM, Aponte J, Quinto L, Martinez-Bauer E, Bruguera M, Sanchez-Tapias JM, Rodes J. Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. Hepatology. 2002;36:986–92.

GESA. The economic cost and health burden of liver diseases in Australia. http://www.gesa.org.au/files/editor_upload/File/GESA%20report%2028032013_web.pdf. 2013. Accessed 15 Aug 2015.

Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. Hepatology. 2009;49:1335–74.
Guechot J, Lasnier E, Sturm N, Paris A, Zarski JP. Automation of the hepascore and validation as a biochemical index of liver fibrosis in patients with chronic hepatitis C from the ANRS HC EP 23 fibrostar cohort. Clin Chim Acta. 2010;411:86–91.
Halfon P, Bacq Y, De Muret A, Penaranda G, Bourliere M, Ouzan D, Tran A, Botta D, Renou C, Brechot MC, Degott C, Paradis V. Comparison of test performance profile for blood tests of liver fibrosis in chronic hepatitis C. J Hepatol. 2007;46:395–402.
Harrison SA, Oliver D, Arnold HL, Gogia S, Neuschwander-Tetri BA. Development and validation of a simple NAFLD clinical scoring system for identifying patients without advanced disease. Gut. 2008;57:1441–7.
Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. Annu Rev Pathol. 2011;6:425–56.
Ho AS, Cheng CC, Lee SC, Liu ML, Lee JY, Wang WM, Wang CC. Novel biomarkers predict liver fibrosis in hepatitis C patients: alpha 2 macroglobulin, vitamin D binding protein and apolipoprotein AI. J Biomed Sci. 2010;17:58.
Huang Y, De Boer WB, Adams LA, Macquillan G, Bulsara MK, Jeffrey GP. Clinical outcomes of chronic hepatitis C patients related to baseline liver fibrosis stage: a hospital-based linkage study. Intern Med J. 2015;45:48–54.
Hui AY, Chan HL, Wong VW, Liew CT, Chim AM, Chan FK, Sung JJ. Identification of chronic hepatitis B patients without significant liver fibrosis by a simple noninvasive predictive model. Am J Gastroenterol. 2005;100:616–23.
Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. Lancet. 2001;357:1069–75.
Islam S, Antonsson L, Westin J, Lagging M. Cirrhosis in hepatitis C virus-infected patients can be excluded using an index of standard biochemical serum markers. Scand J Gastroenterol. 2005;40:867–72.
Jeffrey AW, Huang Y, Adams LA, Macquillan G, Speers D, Rossi E, Bulsara M, Jeffrey GP. Changing Hepascore tests accurately predict increased hepatocellular carcinoma, liver decompensation and liver related death in chronic HCV infection. J Gastroenterol Hepatol. 2015;30:105.
Kalantari H, Hoseini H, Babak A, Yaran M. Validation of hepascore as a predictor of liver fibrosis in patients with chronic hepatitis C infection. Hepat Res Treat. 2011;2011, 972759.
Kawser CA, Iredale JP, Winwood PJ, Arthur MJ. Rat hepatic stellate cell expression of alpha2-macroglobulin is a feature of cellular activation: implications for matrix remodelling in hepatic fibrosis. Clin Sci (Lond). 1998;95:179–86.
Kelleher TB, Mehta SH, Bhaskar R, Sulkowski M, Astemborski J, Thomas DL, Moore RE, Afdhal NH. Prediction of hepatic fibrosis in HIV/HCV co-infected patients using serum fibrosis markers: the SHASTA index. J Hepatol. 2005;43:78–84.
Khoo EY, Stevenson MC, Leverton E, Cross R, Eriksson JW, Poucher SM, Spendlove I, Morris PG, Macdonald IA, Mansell P, Aithal GP. Elevation of alanine transaminase and markers of liver fibrosis after a mixed meal challenge in individuals with type 2 diabetes. Dig Dis Sci. 2012;57:3017–25.
Kitson MT, Kemp WW, Iser DM, Paul E, Wilson JW, Roberts SK. Utility of transient elastography in the non-invasive evaluation of cystic fibrosis liver disease. Liver Int. 2013;33:698–705.
Koda M, Matunaga Y, Kawakami M, Kishimoto Y, Suou T, Murawaki Y. FibroIndex, a practical index for predicting significant fibrosis in patients with chronic hepatitis C. Hepatology. 2007;45:297–306.
Kumar M, Sarin SK, Hissar S, Pande C, Sakhuja P, Sharma BC, Chauhan R, Bose S. Virologic and histologic features of chronic hepatitis B virus-infected asymptomatic patients with persistently normal ALT. Gastroenterology. 2008;134:1376–84.
Lai M, Hyatt BJ, Nasser I, Curry M, Afdhal NH. The clinical significance of persistently normal ALT in chronic hepatitis B infection. J Hepatol. 2007;47:760–7.

Lemoine M, Shimakawa Y, Nayagam S, Khalil M, Suso P, Lloyd J, Goldin R, Njai HF, Ndow G, Taal M, Cooke G, D'alessandro U, Vray M, Mbaye PS, Njie R, Mallet V, Thursz M. The gamma-glutamyl transpeptidase to platelet ratio (GPR) predicts significant liver fibrosis and cirrhosis in patients with chronic HBV infection in West Africa. Gut. 2016;65:1369–76.

Leroy V, Hilleret MN, Sturm N, Trocme C, Renversez JC, Faure P, Morel F, Zarski JP. Prospective comparison of six non-invasive scores for the diagnosis of liver fibrosis in chronic hepatitis C. J Hepatol. 2007;46:775–82.

Leroy V, Sturm N, Faure P, Trocme C, Marlu A, Hilleret MN, Morel F, Zarski JP. Prospective evaluation of FibroTest(R), FibroMeter(R), and HepaScore(R) for staging liver fibrosis in chronic hepatitis B: comparison with hepatitis C. J Hepatol. 2014;61:28–34.

Lok AS, Ghany MG, Goodman ZD, Wright EC, Everson GT, Sterling RK, Everhart JE, Lindsay KL, Bonkovsky HL, Di Bisceglie AM, Lee WM, Morgan TR, Dienstag JL, Morishima C. Predicting cirrhosis in patients with hepatitis C based on standard laboratory tests: results of the HALT-C cohort. Hepatology. 2005;42:282–92.

Mcgary CT, Raja RH, Weigel PH. Endocytosis of hyaluronic acid by rat liver endothelial cells. Evidence for receptor recycling. Biochem J. 1989;257:875–84.

Naveau S, Poynard T, Benattar C, Bedossa P, Chaput JC. Alpha-2-macroglobulin and hepatic fibrosis. Diagnostic interest. Dig Dis Sci. 1994;39:2426–32.

Naveau S, Gaude G, Asnacios A, Agostini H, Abella A, Barri-Ova N, Dauvois B, Prevot S, Ngo Y, Munteanu M, Balian A, Njike-Nakseu M, Perlemuter G, Poynard T. Diagnostic and prognostic values of noninvasive biomarkers of fibrosis in patients with alcoholic liver disease. Hepatology. 2009;49:97–105.

Nguyen-Khac E, Chatelain D, Tramier B, Decrombecque C, Robert B, Joly JP, Brevet M, Grignon P, Lion S, Le Page L, Dupas JL. Assessment of asymptomatic liver fibrosis in alcoholic patients using fibroscan: prospective comparison with seven non-invasive laboratory tests. Aliment Pharmacol Ther. 2008;28:1188–98.

Omata M, Kanda T, Yu M-L, Yokosuk O, Lim S-G, Jafri W, Tateishi R, Hamid SS, Chuang W-L, Chutaputti A, Wei L, Sollano J, Sarin SK, Kao J-H, Mccaughan GW. APASL consensus statements and management algorithms for hepatitis C virus infection. Hepatol Int. 2012;6:409–35.

Ong SY, Dolling L, Dixon JL, Nicoll AJ, Gurrin LC, Wolthuizen M, Wood EM, Anderson GJ, Ramm GA, Allen KJ, Olynyk JK, Crawford D, Kava J, Ramm LE, Gow P, Durrant S, Powell LW, Delatycki MB. Should HFE p.C282Y homozygotes with moderately elevated serum ferritin be treated? A randomised controlled trial comparing iron reduction with sham treatment (Mi-iron). BMJ Open. 2015;5, e008938.

Pankow JS, Boerwinkle E, Adams PC, Guallar E, Leiendecker-Foster C, Rogowski J, Eckfeldt JH. HFE C282Y homozygotes have reduced low-density lipoprotein cholesterol: the Atherosclerosis Risk in Communities (ARIC) Study. Transl Res. 2008;152:3–10.

Papastamataki M, Delaporta P, Premetis E, Kattamis A, Ladis V, Papassotiriou I. Evaluation of liver fibrosis in patients with thalassemia: the important role of hyaluronic acid. Blood Cells Mol Dis. 2010;45:215–8.

Pares A, Deulofeu R, Gimenez A, Caballeria L, Bruguera M, Caballeria J, Ballesta AM, Rodes J. Serum hyaluronate reflects hepatic fibrogenesis in alcoholic liver disease and is useful as a marker of fibrosis. Hepatology. 1996;24:1399–403.

Patel K, Gordon SC, Jacobson I, Hezode C, Oh E, Smith KM, Pawlotsky JM, Mchutchison JG. Evaluation of a panel of non-invasive serum markers to differentiate mild from moderate-to-advanced liver fibrosis in chronic hepatitis C patients. J Hepatol. 2004;41:935–42.

Poynard T, Aubert A, Bedossa P, Abella A, Naveau S, Paraf F, Chaput JC. A simple biological index for detection of alcoholic liver disease in drinkers. Gastroenterology. 1991;100:1397–402.

Raftopoulos SC, George J, Bourliere M, Rossi E, De Boer WB, Jeffrey GP, Bulsara M, Speers DJ, Macquillan G, Ching HL, Kontorinis N, Cheng W, Flexman J, Fermoyle S, Rigby P, Walsh L, Mcleod D, Adams LA. Comparison of noninvasive models of fibrosis in chronic hepatitis B. Hepatol Int. 2012;6:457–67.

Rockey DC, Bissell DM. Noninvasive measures of liver fibrosis. Hepatology. 2006;43:S113–20.
Rosenberg WM, Voelker M, Thiel R, Becka M, Burt A, Schuppan D, Hubscher S, Roskams T, Pinzani M, Arthur MJ. Serum markers detect the presence of liver fibrosis: a cohort study. Gastroenterology. 2004;127:1704–13.
Rossi E, Adams LA, Ching HL, Bulsara M, Macquillan GC, Jeffrey GP. High biological variation of serum hyaluronic acid and Hepascore, a biochemical marker model for the prediction of liver fibrosis. Clin Chem Lab Med. 2013;51:1107–14.
Seeff LB. Natural history of hepatitis C. Hepatology. 1997;26:21S–8.
Sorrell MF, Belongia EA, Costa J, Gareen IF, Grem JL, Inadomi JM, Kern ER, Mchugh JA, Petersen GM, Rein MF, Strader DB, Trotter HT. National Institutes of Health consensus development conference statement: management of hepatitis B. Hepatology. 2009;49:S4–12.
Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, Sulkowski MS, Torriani FJ, Dieterich DT, Thomas DL, Messinger D, Nelson M. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. Hepatology. 2006;43:1317–25.
Tiggelman AM, Linthorst C, Boers W, Brand HS, Chamuleau RA. Transforming growth factor-beta-induced collagen synthesis by human liver myofibroblasts is inhibited by alpha2-macroglobulin. J Hepatol. 1997;26:1220–8.
Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, Lok AS. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology. 2003;38:518–26.
Wells AF, Lundin A, Michaelsson G. Histochemical localization of hyaluronan in psoriasis, allergic contact dermatitis and normal skin. Acta Derm Venereol. 1991;71:232–8.
Wu SD, Wang JY, Li L. Staging of liver fibrosis in chronic hepatitis B patients with a composite predictive model: a comparative study. World J Gastroenterol. 2010;16:501–7.
Zarski JP, Sturm N, Guechot J, Paris A, Zafrani ES, Asselah T, Boisson RC, Bosson JL, Guyader D, Renversez JC, Bronowicki JP, Gelineau MC, Tran A, Trocme C, De Ledinghen V, Lasnier E, Poujol-Robert A, Ziegler F, Bourliere M, Voitot H, Larrey D, Rosenthal-Allieri MA, Fouchard Hubert I, Bailly F, Vaubourdolle M. Comparison of nine blood tests and transient elastography for liver fibrosis in chronic hepatitis C: the ANRS HCEP-23 study. J Hepatol. 2012;56:55–62.
Zeng MD, Lu LG, Mao YM, Qiu DK, Li JQ, Wan MB, Chen CW, Wang JY, Cai X, Gao CF, Zhou XQ. Prediction of significant fibrosis in HBeAg-positive patients with chronic hepatitis B by a noninvasive model. Hepatology. 2005;42:1437–45.
Zhou K, Gao CF, Zhao YP, Liu HL, Zheng RD, Xian JC, Xu HT, Mao YM, Zeng MD, Lu LG. Simpler score of routine laboratory tests predicts liver fibrosis in patients with chronic hepatitis B. J Gastroenterol Hepatol. 2010;25:1569–77.

Model for End-Stage Liver Disease (MELD) Score as a Biomarker

3

Deepika Devuni and Jawad Ahmad

Contents

D. Devuni • J. Ahmad (✉)
Division of Liver Diseases and Recanati-Miller Transplantation Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA
e-mail: jawad.ahmad@mountsinai.org; javbob@hotmail.com

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_30

Abstract

End-stage liver disease (ESLD) due to cirrhosis carries a high mortality. Previous methods to quantify the risk of death in these patients were subjective. The model for end-stage liver disease (MELD) score was developed and is an accurate biomarker of 90-day mortality in patients with ESLD, essentially measuring how sick a patient is. The MELD score incorporates serum bilirubin, creatinine, and INR in a mathematical formula. Since 2002, the MELD score has been used to prioritize deceased donor organ allocation for patients listed for liver transplantation (LT) in the USA. The use of the MELD allocation system has resulted in sicker patients being transplanted with decreased waiting time, thereby decreasing the death rate on the LT waiting list, without an adverse effect on posttransplant outcome. The MELD score has been adopted as a biomarker with good effect in other situations where patients with ESLD have a high risk of dying such as surgery, alcoholic hepatitis, acute liver failure, and variceal bleeding. Since the MELD score was introduced, there have been several modifications that may have increased effectiveness in certain situations. The MELD score is not an accurate biomarker for the risk of death from liver cancer and some other conditions, and hence for the purposes of liver allocation on the transplant list, an exception to the calculated MELD score can be given.

Keywords

Model for end-stage liver disease (MELD) score • End-stage liver disease • Liver transplantation • Organ allocation

Abbreviations

AH	Alcoholic hepatitis
CTP	Child-Turcotte-Pugh
DF	Discriminant function
ESLD	End-stage liver disease
FHF	Fulminant hepatic failure
HCC	Hepatocellular carcinoma
HCV	Hepatitis C
HRS	Hepatorenal syndrome
ICU	Intensive care unit
LT	Liver transplantation
MELD	Model for end-stage liver disease
OPO	Organ procurement organization
OPTN	Organ procurement and transplantation network
PSE	Portosystemic encephalopathy
TIPS	Transjugular intrahepatic portosystemic shunt
UNOS	United Network for Organ Sharing

Key Facts of the MELD Score

1. The MELD score is a mathematical formula that uses readily available blood tests to predict the severity of a patient's liver disease.
2. The MELD score is used to determine where patients are on the waiting list for liver transplantation in the USA – a higher score puts you higher on the list.
3. The MELD score ensures sicker patients are transplanted first.
4. The MELD score can be used in other liver conditions to predict how sick patients are.
5. The MELD score does not predict how well patients will do after liver transplantation.

Introduction

End-stage liver disease (ESLD) due to cirrhosis is the 12th leading cause of mortality in the USA according to the Centers for Disease Control (CDC). Patients with cirrhosis can be asymptomatic for many years during the compensated phase, but patients with decompensated cirrhosis have developed complications related to portal hypertension in the form of ascites, portosystemic encephalopathy, and variceal bleeding and are at risk of developing hepatocellular carcinoma (HCC). Patients who develop complications of portal hypertension and/or HCC are candidates for liver transplantation (LT).

Liver transplantation typically leads to 70% 5-year survival, but the main restriction is the lack of deceased donor organs. In the USA the number of patients waiting for LT (approximately 15,000) and transplants performed annually (approximately 6,000) has not changed for a decade, meaning a significant number of patients that are listed for LT will not get transplanted before they die or are removed from the list for being too sick to transplant. An equitable method to prioritize deceased donor organs is therefore of paramount importance. The federal government and the transplant community recognized this in the late 1990s, and this led to the development of the model for end-stage liver disease (MELD) score which has been used to prioritize potential recipients for LT since February 2002.

This paper will illustrate the strengths and weaknesses of the MELD score as a biomarker in patients with ESLD and its adaptation and modification for use in other liver diseases.

Natural History of Liver Disease

End-stage liver disease is typically due to viral hepatitis (B or C), fatty liver disease, alcoholic liver disease, autoimmune liver disease, metabolic liver diseases, and cryptogenic cirrhosis. The disease progression is very variable with some patients only developing inflammation without significant fibrosis, but a minority of patients will develop progressive fibrosis and eventually cirrhosis. Patients with cirrhosis can

often be asymptomatic and therefore undiagnosed unless they have been followed regularly. In addition, the compensated phase of cirrhosis can last for many years even when there are laboratory abnormalities. However, decompensation, defined by the development of ascites, variceal bleeding, or portosystemic encephalopathy (PSE), usually signals a more rapid progression of disease with mortality after the first decompensating event as high as 50% at 5 years (Fattovich 1997).

The HALT-C group followed a group of 1,050 subjects with chronic hepatitis C (HCV) and advanced fibrosis and determined that the incidence of cirrhosis was 9.9% per year (Dienstag 2011). In the study by Fattovich et al. (1997), 384 chronic HCV patients with cirrhosis were followed for more than 10 years, and the 5-year probability for hepatic decompensation was 18%, and 5-year survival was 91%. In a study comparing alcoholic cirrhosis and HCV, the risk of hepatic decompensation and mortality was similar, and importantly alcohol abstinence even in patients who had already developed cirrhosis improved the survival benefit (Toshikuni 2009).

Once decompensation has occurred, standard medical therapy can alleviate symptoms such as diuretics for ascites, endoscopic management of varices, and lactulose for PSE, but it does not reverse the pathologic process of cirrhosis. Such patients are therefore candidates for LT. With the high burden of liver disease, particularly viral hepatitis in the USA, the number of potential LT candidates exceeds the number of donor organs available. Hence the policy governing the allocation of these scarce organs has been under scrutiny ever since the early days of LT.

Allocation of Organs Prior to MELD

The allocation of deceased donor liver allografts was based on a system that emphasized patients' waiting time and hospitalization status separated into three main categories. Patients admitted to the intensive care unit (ICU) received priority over admitted patients in the hospital followed by patients who were ambulatory. The transplant community met to formulate the minimal criteria for placing adult patients on the LT waiting list (Lucey et al. 1997). They suggested that patients with all causes of cirrhosis with a Child-Turcotte-Pugh score of ≥ 7 or the presence of portal hypertensive gastrointestinal bleeding would qualify to be on the waiting list. The assessment of severity of liver disease was based on the Child-Turcotte-Pugh score.

Child-Turcotte-Pugh (CTP) Score

The CTP score was initially developed for assessing the severity of liver disease. In 1964, Child and Turcotte published a classification system as a tool to determine the preoperative risk of portosystemic shunt surgery for patients with variceal bleeding. It included five factors – encephalopathy, serum bilirubin, nutritional status, ascites, and serum albumin (Child and Turcotte 1964). Pugh et al. (1973) modified the score by replacing nutritional status with prothrombin time. They also added scores

Table 1 Child-Turcotte-Pugh scoring system for patients with ESLD

	1 point	2 points	3 points
Serum bilirubin	<2 mg/dl	2–3 mg/dl	>3 mg/dl
Serum albumin	>3.5 g/dl	2.8–3.5 g/dl	<2.8 g/dl
Ascites	Absent	Controlled with medications	Refractory
Encephalopathy	Absent	Medically controlled	Poorly controlled
INR	<1.7	1.7–2.2	>2.2

Table 2 Minimal listing criteria for liver transplantation prior to the MELD score

Status	Definition
1	Life expectancy of less than 7 days without transplantation:
	1. Fulminant hepatic failure
	2. Primary graft nonfunction within 7 days of LT
	3. Hepatic artery thrombosis less than 7 days after LT
	4. Acute decompensated Wilson disease
2A	In ICU with a CTP score >10 with unresponsive active variceal hemorrhage or hepatorenal syndrome or refractory ascites or hepatic hydrothorax or stage 3 and 4 encephalopathy
2B	Inpatients with a CTP score of ≥10 or a CTP score of ≥7 and either unresponsive active variceal hemorrhage or hepatorenal syndrome or spontaneous bacterial peritonitis and refractory ascites or hepatic hydrothorax
3	Patients needing continuous medical care, with a CTP score of 7 but not meeting criteria for status 2B
7	Temporarily inactive due to various reasons

ranging from 1 to 3 for each factor based on severity. They used the modified score to classify patients into A (5–6 points), B (7–9 points), or C (10 or more points) categories based on the cumulative points (Table 1). This scoring system was then used to assess the outcomes of surgery in patients with cirrhosis undergoing esophageal transection for bleeding varices. Patients with CTP class C had the highest mortality. The CTP score was included in the liver organ allocation as part of minimal listing criteria (Table 2).

The main disadvantage of using the CTP score was that the severity of ascites and encephalopathy are subjective, and it does not take into account renal function which is often abnormal in patients with more severe ESLD.

Development of MELD

The allocation of donor organs for LT is based on the availability of organs at the local organ procurement organization (OPO). There have always been geographic disparities in waiting time within the different areas in the USA which have increased as the number of transplant centers has increased despite the National Organ Transplant Act of 1984 which was meant to ensure equitable distribution of organs.

In 1998, the United States Department of Health and Human Services issued the "Final Rule" regulation (OPTN 1999). The principles of the "Final Rule" mandated that the sickest patients should get transplanted first without limitation of geographic area. It also recommended that a system be developed to standardize the criteria to place a patient on the waiting list and use factors to assess severity of liver disease with less subjective variability. The effect of disease severity and waiting time had been illustrated in a study by Freeman and Edwards (2000), which reviewed the 16,414 patients that were added to the waiting list from January 1997 to December 1997. They demonstrated that disease severity at the time of listing had a significant impact on mortality whereas waiting time did not. What was required was an accurate biomarker of liver disease severity that could measure the risk of dying with ESLD.

The precursor of the MELD score was based on article by Malinchoc et al. (2000) in which they were developing a statistical model to predict patient survival in patients undergoing elective transjugular intrahepatic portosystemic shunt (TIPS). A total of 231 patients were included from four transplant centers in the USA. The median survival time post TIPS was 1.4 years. In univariate analysis increasing levels of ascites, hepatic encephalopathy, Child-Pugh class and Child-Pugh score, bilirubin, creatinine, and INR significantly had a negative effect on survival. Increasing albumin level had a positive effect on survival. In multivariate analysis, they found serum creatinine, serum bilirubin, INR, and the cause of cirrhosis to be independent risk factors for mortality. These factors were then weighted to come up with a disease severity index that was termed the model for end-stage liver disease (MELD) score. The same group then examined the disease severity index in patients waiting for LT (Kamath et al. 2001). The formula for the modified MELD score is $3.8[\log_e$ serum bilirubin (mg/dL)] + 11.2 [$\log_e$ INR] + 9.6 [$\log_e$ serum creatinine (mg/dL)] + 6.4. The study initially included 282 patients hospitalized for complications of liver disease. Patient survival was assessed as the interval from the day of hospitalization to the last day of follow-up or death. The C-statistic for prediction of 3-month survival by MELD score was 0.87. They studied patients in the ambulatory setting and found a C-statistic of 0.87 and 0.8, respectively. The study also demonstrated that complications of portal hypertension such as ascites, variceal bleeding, or encephalopathy did not add to the C-statistic of the MELD score. In conclusion, the group felt that MELD score was better than CTP score and had less variability. They suggested that it followed the principle of the "Final Rule."

For the purposes of deceased donor organ allocation, patients with ESLD waiting for LT are each given a MELD score based on their laboratory parameters. The score increases as the severity of their disease worsens and so is checked periodically so that they can move up the list. The score starts at 6, since the lowest value of each integer is set at 1, and is typically capped at 40 since the 3-month mortality at this score is more than 80% (Fig. 1).

The MELD score was adopted as the method for prioritizing organs for deceased donor liver allocation on February 27, 2002. After the application of the MELD score, several studies showed a decrease in transplant waiting list mortality. Weisner et al. (2003) examined 3,437 liver transplant patients added to the list between

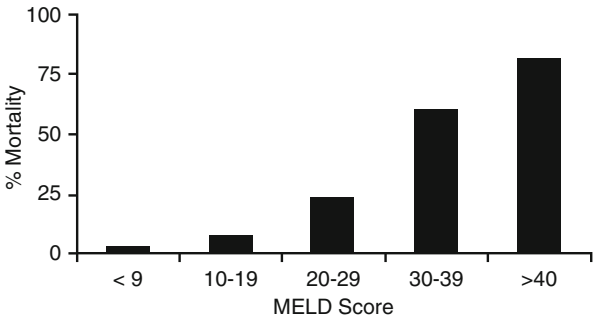

Fig. 1 Three-month mortality based on listing MELD in patients on the OPTN waiting list (From SRTR/OPTN annual report 2003 (www.srtr.org))

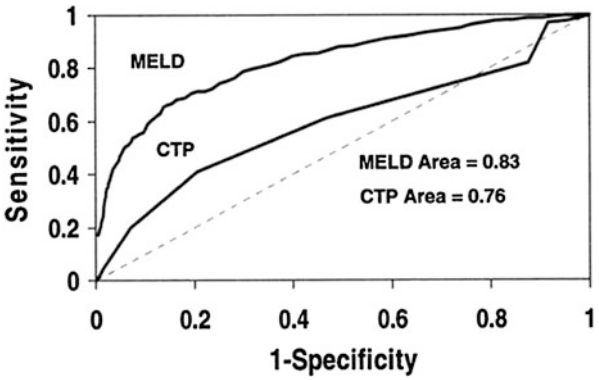

Fig. 2 Graph comparing MELD score and CTP score in assessing 90-day mortality in patients with ESLD

November 1999 and 2001. Four hundred twelve patients died during the 3-month follow-up period. Patients with MELD $\geq$40 had a mortality of 71%. The C-statistic for MELD score was 0.83 as compared to CTP score which was 0.76 (Fig. 2).

Data from the United Network for Organ Sharing (UNOS) had demonstrated that the number of patients waiting for LT had been steadily increasing for the 8 years prior to introduction of the MELD score (Fig. 3). Freeman et al. (2004) compared the rates of transplant listing, transplants, deaths, and removals between the year of MELD implementation and the year before. They observed a 12% decrease in the waiting list registrants as patients did not get an advantage of time on the list. There was also 3.5% decrease in waiting list mortality in the MELD era as compared to the year before as the sickest patients were transplanted first. Ahmad et al. (2007a) studied the impact of MELD allocation on US veterans undergoing LT in the Veterans' Healthcare System. A total of 207 patients were included in the study with 83 patients transplanted pre-MELD and 124 in the MELD era. The mean waiting time decreased from 461 days (pre-MELD) to 252 days (MELD era) ($P = 0.004$), and the mean MELD score at LT increased to 23.4 (MELD era) compared to 20.3 (pre-MELD) ($P = 0.01$), concluding that implementation of the MELD system led to sicker veterans being transplanted with shorter waiting times.

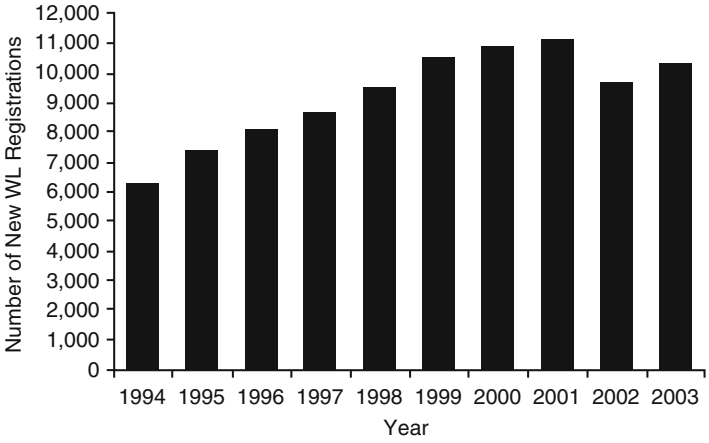

Fig. 3 New liver waiting list registrations in the USA from 1994 to 2003 (From 2004 OPTN/SRTR annual report, Table 1.5)

This shorter waiting time of sicker patients should translate into reduced death on the waiting list. In a study from Germany, Quante et al. (2012) reviewed the effect of MELD on wait list mortality. Wait list mortality was decreased from 18% to 10% ($p = 0.04$). The mean MELD score at allocation increased from 16.4 to 22.7 ($P = 0.007$), and the 90-day and 1-year survival post LT was found to remain stable at 90%.

MELD and Impact on Liver Transplantation

One of the main concerns and opposition of transplanting the "sickest first" was the impact it might have on early graft and patient survival since sicker patients presumably would not do as well posttransplant. In a study by Merion et al. (2005), survival benefit was assessed at various MELD levels. The survival benefit was defined as the difference between survival with or without transplant. Even if patients with a very high MELD score did not do as well after LT as patients with lower MELD scores, this would be offset by the very high mortality these patients have without LT. They demonstrated that the survival benefit at a MELD score of 40 was 96%. This suggests that high MELD score patients should get transplanted first, and the fact they did no worse than lower MELD score patients means that the MELD score is *not* a good biomarker for posttransplant outcome. Importantly, they also demonstrated that patients with MELD score <15 had worse outcomes with transplant, meaning that most patients with a low MELD score are better off waiting for LT until they become sicker.

The United Network for Organ Sharing has established 11 geographic regions for administrative purposes. There are 58 organ procurement areas which are responsible for retrieving organs and assigning them locally, then regionally, and

finally nationally depending on the MELD score of patients on the waiting list (which is subdivided by blood type). Despite the mandate that geography should not influence waiting time and MELD score at transplant, major differences still exist. Since some patients were still being transplanted in some regions at a low MELD score, with potentially worse outcomes, UNOS proposed a change in liver allocation: "Share 15" in January 2005. They proposed that the distribution sequence for a donor liver would be as follows: (1) local status 1, (2) OPTN region status 1, (3) local MELD $\geq$15, (4) OPTN region MELD $\geq$15, (5) local MELD $<$15, (6) OPTN region $<$15, (7) national status 1, and (8) national any MELD (Pomfret et al. 2007). This led to 36% decrease in transplants in MELD $<$15 and the proportion of transplants to recipients with MELD $\geq$15 increased in all geographic areas. Overall this improved the outcomes after LT, but a "MELD exception" system exists where patients with low MELD scores can be reviewed and appealed for a higher MELD score. A study by Bittermann et al. (2012) analyzed 452 MELD exceptions, 197 patients received a transplant, and 80% of these patients had MELD $<$15.

In spite of the Share 15, there is a disparity among various UNOS regions in terms of median MELD score at listing and transplant and time to transplant. Ahmad et al. (2007b) analyzed if MELD score at transplantation and waiting time of liver transplant recipients differs by transplantation center volume. They showed that despite having lower MELD scores, recipients at high-volume centers also experienced shorter waiting times (median waiting time, 69 days vs. 98 days, and 94 days at medium- and low-volume centers, respectively; $P < 0.001$).

In an ongoing effort to improve organ allocation, UNOS implemented "Share 35" on June 18, 2013. According to this rule, deceased donor livers are offered to regional candidates with MELD $\geq$35 before local candidates with MELD $<$35. Massie et al. (2015) compared the liver distribution and mortality in the first year of "Share 35." During this time, the proportion of deceased donor liver transplants (DDLTs) allocated to recipients with MELD $\geq$35 increased from 23.1% to 30.1% ($p < 0.001$). The proportion of regional shares increased from 18.9% to 30.4% ($p < 0.001$). There was a 30% decrease in wait list mortality of patients with MELD $>$30 but no difference in patients whose baseline MELD scores were lower. There were less discards of livers and no change in cold ischemia time.

MELD Use in Other Countries

In Europe, some countries have now adopted the MELD allocation policy including Eurotransplant in December 2006 (Germany, the Netherlands, Belgium, Luxembourg, Austria, Slovenia, and Croatia) Dutkowski et al (2010), North Italian Transplant in March 2003, Swiss Transplant in July 2007, and "l'Etablissement Français des Greffes" in France in March 2007. By contrast, several other European countries, such as Spain, Sweden, Finland, Norway, Denmark, Iceland, and the United Kingdom, still prefer to distribute their organs through a center-directed system.

In a study by Dutkowski et al. (2011), from Switzerland, they compared the first 100 transplants before and after implementation of MELD allocation. There was a decrease in waiting list mortality from 386 versus 242 deaths per 1,000 patient-years ($P < 0.0001$). It also led to transplantation of sicker patients, MELD 13.5 vs. 20 ($p = 0.003$), but the cost of transplantation was higher in the post-MELD era although patient survival was stable in both groups.

In another study from Germany, Benckert et al. (2011) studied 142 patients and found that wait list mortality had decreased, but 90-day mortality post LT did not change. They also confirmed that the MELD score is not a good biomarker of prognosis after LT. Similar findings were noted in Brazil, where MELD allocation was introduced in 2006 (Freitas et al. 2010).

MELD and Prognosis of Cirrhosis

Since the initial studies to develop MELD, multiple studies have evaluated the validity of the MELD score to assess the prognosis of liver disease. Botta et al. (2003) reviewed 129 patients with cirrhosis, and they found that the MELD score was a good predictor of short- and long-term survival and was equivalent to the CTP score. Papatheodoridis et al. (2005) compared MELD, CTP score, and creatinine modified CTP scores in decompensated cirrhosis. The accuracy of MELD was similar to the modified CTP score to predict short-term mortality, and MELD was better for long-term mortality. A large study of 1,611 patients with a spectrum of liver diseases found that patients with alcoholic liver disease had a higher 1-year and 5-year mortality than patients with alcoholic liver disease with hepatitis C, hepatitis C, or other causes of liver disease. The MELD score predicted increased mortality, with each unit increase in the MELD score predicting a 4–9% increase in mortality ($P = 0.0001$). The ROC curve C-statistic for the MELD as a predictor of 1-year mortality was 0.80 for all patients. They also found hepatic encephalopathy to be an independent predictor of mortality (Said et al. 2004). Similar findings were noted in 312 cirrhotic patients admitted to an intensive care unit. The overall mortality was 65.1%. The SOFA score (AUC 0.83) and MELD (AUC 0.81) were better predictors of mortality than traditional scores used in critically ill patients such as APACHE II (0.78) or Child-Pugh score (AUC 0.72). The authors concluded that cirrhotics with $\geq$3 organ system failure had 90% mortality (Cholongitas et al. 2006). In a study from India, 102 patients with cirrhosis were studied. They compared MELD, CTP score, and creatinine modified Child-Pugh score (CrCTP). The MELD was superior to CTP for predicting 3-month [C-statistic and 95% confidence interval, 0.967 (0.911–0.992) vs. 0.884 (0.806–0.939)] and 6-month [0.977 (0.925–0.996) vs. 0.908 (0.835–0.956)] mortality ($P = 0.05$), while CrCTP [0.958 (0.899–0.988)] was better than CTP for predicting 3-month mortality ($P = 0.02$). Serum creatinine (hazard ratio 4.43, $P < 0.0001$) was a strong independent predictor of mortality (Chawla et al. 2011).

Variability in MELD Calculation

The advantage of the MELD score over the CTP score is the objective measure in the variables that make up the MELD score. However, several studies have demonstrated that there can be interlaboratory differences in the calculation of MELD. Trotter et al. (2004) first demonstrated this when they compared the same blood sample in three different laboratories. They found that there was a statistical difference in MELD in one laboratory compared to the other two (MELD of 14 versus 17, $P < 0.03$). Most of the difference in the MELD score was due to the measurement of INR. This led to patients being listed with higher priority points. The same group looked at a larger number of laboratories and analyzed the interlaboratory variation in INR and if the differences would translate into clinically relevant changes in MELD score. They divided the samples in five different groups and INR ranged from (1.2–2) in sample 1 to (2.4–5.1) in sample 5. The variability of INR increased as the mean INR increased ($p = 0.0174$). Differences in MELD score were as high as 7 points (Trotter et al. 2007).

A similar effect on MELD scores with creatinine levels using different assays has been noted. Analysis of 403 samples from 158 patients concluded that the variability in creatinine measurement increased with rising serum bilirubin concentration, with a MELD variation of 3–7 points. A MELD score $\geq$25 was associated with the greatest variability. The authors concluded that there was poor correlation in creatinine scores with rising bilirubin and this may affect the MELD scores (Cholongitas et al. 2007a). These differences were also noted by two other studies (Schouten et al. 2012; Kaiser et al. 2014) as have gender differences since women with liver disease have a lower glomerular filtration rate for the same creatinine value. This may lead to women not getting priority on the waiting list (Cholongitas et al. 2007b).

MELD in Other Conditions

Since the adoption of the MELD score for prioritizing deceased donor liver allocation, it has been studied in various other situations involving patients with liver disease.

Surgical Risk in Patients with Cirrhosis

Patients with cirrhosis have a high risk of morbidity and mortality with any surgical procedure. Surgeons several decades ago were aware that the risk of mortality in cirrhotic patients undergoing cholecystectomy was as high as 25% (Aranha et al. 1982). Investigators then noted that several factors such as CTP score >7, presence of ascites, and elevated serum creatinine were associated with high mortality after surgery in patients with cirrhosis (Ziser et al. 1999). Teh et al. (2007) studied the short-term and long-term mortality risks in patients with cirrhosis who underwent various surgical procedures and specifically examined the effectiveness

of the MELD score as a biomarker. They looked at 772 patients undergoing abdominal ($n = 586$), orthopedic ($n = 109$), or cardiovascular ($n = 79$) surgery. The MELD score, anesthesia class, and patient age predicted mortality at 30 and 90 days, 1 year, and long-term follow-up to 20 years independent of the type of surgery. Surgery involving the liver in patients with underlying liver disease is another situation where an accurate preoperative biomarker is useful to stratify surgical risk. Cucchetti et al. (2006) studied the effectiveness of the MELD score as a biomarker on post hepatectomy outcomes in patients with HCC. One hundred fifty four patients undergoing HCC resection in a tertiary care setting were followed. Eleven patients had liver decompensation leading to death or requiring LT. A MELD score >11 was predictive of postoperative liver failure (area under the curve [AUC] = 0.92, 95% confidence interval [CI] (0.87–0.96); sensitivity, 82%; specificity, 89%). Cirrhotic patients with MELD score <9 had no postoperative liver failure and low morbidity (8.1%). Other studies have found similar results. A MELD score >8 was highly predictive of mortality in patients undergoing HCC resection (Hsu et al. 2009), and a MELD score >15 or greater had significant mortality after tricuspid repair or replacement (Ailawadi et al. 2009). Northup et al. (2005) analyzed 131 patients who underwent 140 non-transplant surgical procedures and found an overall 30-day postoperative mortality of 16.4%. They demonstrated that the mean MELD score in patients who died (24.8, 20.4–29.3) was significantly higher than survivors (16.2, 14.2–18.2), ($P = 0.0001$).

Alcoholic Hepatitis

Alcoholic hepatitis (AH) is characterized by acute or acute on chronic inflammation in the liver due to current alcohol consumption. Symptoms are variable but range from asymptomatic to fever, profound hyperbilirubinemia, and fatigue. Mortality can be as high as 50% (Menon et al. 2001). The earliest biomarker in this disease is the discriminant function (DF) which has been used to predict short-term survival in patients with AH and is calculated by the following equation: $\mathrm{DF} = 4.6[\text{Prothrombin Time in seconds} - \text{control Prothrombin Time}] + \text{serum bilirubin}(\mathrm{mg/dL})$. Maddrey et al. (1978) demonstrated that a DF score ≥32 predicts significant mortality and was an indication for the use of corticosteroids in this group of patients with improvement in survival. Several studies have used the MELD score as a biomarker to prognosticate in patients with AH. Dunn et al. (2005) conducted a retrospective study of 73 patients with AH and found MELD was the only independent predictor of mortality, with a MELD score of 21 having a sensitivity of 75% and a specificity of 75% in predicting 90-day mortality. The C-statistic comparing the prognostic validity of MELD and DF in AH was comparable for 30-day as well as 90-day mortality. The MELD score was better than the CTP score and DF in predicting mortality in patients with AH in a study of 202 patients. MELD scores were recorded at two time points, including admission and at the first week, along with the interval change in score. All three of these factors were found to be independently associated with in-hospital mortality. The first week MELD score cut

off of 20 had the best sensitivity and specificity in predicting mortality (Srikureja et al. 2005). A more recent study by Goyal et al. (2014) compared MELD, DF, CTP, and the Lille score for predicting the short-term mortality in patients with AH. A MELD score >14 at admission and >12 at day 7 had high sensitivity and specificity in predicting short-term mortality.

Acute Liver Failure

Patients with acute liver failure who are candidates for LT in the USA are listed as status 1. This means they get priority over the entire list of patients with a MELD score. According to UNOS, patients listed as status 1 must fulfill one of the following criteria: (1) fulminant hepatic failure (FHF), defined as the onset of hepatic encephalopathy within 8 weeks of the first symptoms of liver failure with no pre-existing liver disease; (2) nonfunction of a transplanted liver within 7 days of implantation; (3) hepatic artery thrombosis in a transplanted liver within 7 days; and (4) acute decompensated Wilson disease. Patients must fulfill one of the above criteria in addition to being hospitalized in the ICU with a life expectancy to be less than 7 days to be qualified for the emergent listing (Table 2). The transplant-free survival in patients with FHF is only 43% (Ostapowicz and Lee 2000).

A large study evaluated the ability of the MELD score at listing to predict pretransplant and posttransplant survival for patients listed as status 1 (Kremers et al. 2004). The investigators examined 720 patients listed for LT with FHF. They were divided into two groups, FHF associated with acetaminophen (FHF-A) and FHF associated with non-acetaminophen (FHF-NA) causes. There were two other small diagnostic groups: patients with primary nonfunction (PNF) and hepatic artery thrombosis (HAT). They demonstrated that patients with FHF-NA had the poorest survival and the MELD score had a negative correlation with survival. These people demonstrated most benefit from transplant.

Yantorno et al. (2007) compared the efficacy of King's College criteria, Clichy's criteria, and MELD score in adults with FHF. In the 120 study patients, MELD had the highest C-statistic of 0.95 and was superior to King's College criteria. A MELD score >18 on day 2 of acetaminophen ingestion was associated with development of hepatic encephalopathy (C-statistic 0.92) in patients in the US acute liver failure study group. The difference in MELD on the first day and at the onset of encephalopathy (ΔMELD) was associated with poor prognosis, and MELD itself was not a predictor of survival (Schmidt and Larsen 2007); however, this was not seen in an Indian cohort of FHF patients (Dhiman et al. 2007).

In a more recent study of the UNOS database, ESLD patients with the highest MELD scores were compared to patients listed as status 1. The study included 52,459 candidates (status 1 candidates, $n = 2{,}128$; ESLD candidates, $n = 50{,}331$) aged ≥18 years who were listed for deceased donor LT between September 1, 2001, and December 31, 2007. Out of the 2,128 patients listed as status 1, 485 had acetaminophen-induced liver failure, and the remaining 1,643 were non-acetaminophen induced. The study showed that patients with MELD scores

>40 had significantly higher wait list mortality risk than status 1 candidates. The authors suggested that patients with ESLD with MELD >40 should get priority over status 1 patients. ESLD patients with MELD scores 36–40 had similar wait list mortality risk as status 1 candidates, and hence they concluded that they should be prioritized equally rather than sequentially. Post-LT survival was similar among status 1 and all groups of ESLD candidates. MELD was a significant independent predictor of wait list mortality in the acetaminophen status 1 subgroup (Sharma et al. 2012).

Variceal Bleeding

Gastroesophageal varices develop in about 50% of patients with cirrhosis and typically correlate with severity of the disease. The annual rate of esophageal variceal bleeding (EVB) is 5–15% (Garcia Tsao et al. 2007) with a 6-week mortality of 15–20% and as high as 30% patients with severe decompensated liver disease (CTP score C) (Villanueva et al. 2006). Chalasani et al. (2002) conducted a study to compare the MELD score with the CTP score as a prognostic marker in patients with acute variceal bleeding. In-hospital and 1-year mortality rates were 14.2% and 27%, respectively. The C-statistic for MELD score to predict mortality was 0.82, significantly higher than the CTP score.

Decompensation During Interferon Therapy of HCV Cirrhosis

A recent study of patients receiving pegylated interferon and ribavirin treatment for HCV-related cirrhosis demonstrated that hepatic decompensation was seen in 36.8% of patients during treatment and the MELD score was independently predictive of decompensation with a MELD score >14 associated with 83% chance of worsening of liver disease while on treatment (Dultz et al. 2013).

Hepatorenal Syndrome

Hepatorenal syndrome (HRS) is defined as the development of impaired renal function in patients with cirrhosis or other liver disorders and is characterized by renal vasoconstriction. HRS is classified into two clinical subtypes. Type 1 is defined as a doubling of the serum creatinine concentration (above 2.5 mg/dL) and reduction of creatinine clearance (CrCl) by 50% (or <20 mL/min) in less than 2 weeks. It is associated with very poor outcome with 1-month mortality exceeding 50%. Type 2 is defined by an increase in serum creatinine level to >1.5 mg/dL (or CrCl <40 mL/min) and a urine sodium level <10 mmol/L. Type 2 HRS has a less progressive course but still has a 6-month mortality of 50% (Arroyo et al. 1996).

Alessandria et al. (2005) studied 105 patients ($n = 41$ type 1, $n = 64$ type 2) with HRS. They demonstrated that the MELD score was an independent predictor of

mortality. All patients with type 1 HRS had a high MELD score ($\geq$20) and had a median survival of 1 month. The survival in type 2 HRS patients correlated with their MELD score. Patients with a MELD score >20 had a median survival of 3 months and patients with MELD <20 had a survival of 11 months.

MELD Variations

ΔMELD

Variation of MELD over time has been suggested as an important biomarker in patients with ESLD as it relates to residual liver function. Merion et al. (2005) followed serial MELD scores in patients listed for transplant. Patients with MELD score increases greater than five points over 30 days had a threefold greater wait list mortality risk than those for whom MELD scores increased more gradually ($P < 0.0001$). For any MELD score, the magnitude of change in the last 30 days was considered an independent risk factor for mortality. Similar results were noted when comparing the C-statistic of MELD, ΔMELD, and CTP score in 351 cirrhotic patients. ΔMELD was superior to initial MELD and CTP scores to predict intermediate term outcome in patients with advanced cirrhosis (Huo et al. 2005).

MELD Sodium (MELDNa)

Hyponatremia is a considered a poor prognostic marker in patients with cirrhosis. It is associated with ascites and HRS and predicts increased mortality in cirrhosis. Several studies have demonstrated that in patients with a relatively low MELD score (<21), hyponatremia (Na <135) and persistent ascites were independent predictors of mortality (Heuman et al. 2004; Dawwas et al. 2007). Patients with hyponatremia but a low MELD score are therefore disadvantaged by the current MELD score-based allocation system. Ruf et al. (2005) investigated the prognostic value of adding serum sodium to the MELD score in 262 patients with ESLD. The risk of death across all MELD scores was higher with hyponatremia than those without, with a C-statistic for MELD with serum sodium of 0.905. In addition, a serum sodium <126 meq/l at listing or while listed for transplantation is an independent predictor of 3- and 6-month mortality (Biggins et al. 2005), and there was a significant interaction between MELD score and the serum sodium concentration in 6,769 liver waiting list registrants, with a more pronounced effect of serum sodium on patients with a lower MELD score (Kim et al. 2008).

Based on these findings, the MELD score was modified to incorporate the serum sodium concentration (Biggins et al. 2006; Kim et al. 2008). The MELDNa is calculated by: $\text{MELDNa} = \text{MELD} - \text{Na} - [0.025 * \text{MELD} * (140 - \text{Na})] + 140$, for sodium concentrations between 125 and 140 mEq/L. The MELDNa was better at predicting mortality in patients listed for LT compared to the MELD score (Kim et al. 2008). Several other investigators found that MELDNa was a superior biomarker of mortality compared to the standard MELD score in other situations,

including acute decompensated hepatitis (Hsu et al. 2010), hepatocellular carcinoma (Huo et al. 2008), and cirrhotic patients undergoing surgery (Cho et al. 2011).

iMELD

Another modification of the MELD score is the iMELD. MELD, age, and serum sodium were noted to be independent risk factors for mortality in a cohort of patients undergoing TIPS, and these three factors were incorporated into an integrated MELD (iMELD) by a European group. The iMELD was better than the original MELD in predicting 12-month mortality and was validated in a sample of 451 patients with cirrhosis on the waiting list for LT with increased auROC (+8%) and likelihood ratio statistic (from 41.4 to 82.0) (Luca et al. 2007). The iMELD was a better prognostic model for outcome prediction in patients with decompensated cirrhosis compared to MELD and MELDNa (Jiang 2008).

MELD-XI

Cardiohepatic syndrome is described as the development of liver dysfunction which can subsequently lead to cirrhosis in patients with heart failure (van Deursen 2010). Many of these patients are on anticoagulation. Therapeutic anticoagulation will artificially increase the INR and confer an advantage to these patients under the existing system of organ allocation. Hence, an alternative score was developed, called MELD-XI (MELD excluding INR), by normalizing to the same scale as MELD but omitting INR. The formula for MELD-XI is MELD-XI $=$ 5.112 ln (bilirubin) + 11.76 ln (creatinine) + 9.44. MELD-XI was comparable to MELD as a predictor of pretransplant 90-day mortality (Heuman et al. 2007). C-statistics for MELD and MELD-XI were comparable in patients with cholestatic liver diseases (0.905 ± 0.030 vs. 0.894 ± 0.031) as well as non-cholestatic causes of cirrhosis (0.857 ± 0.016 vs. 0.843 ± 0.016). In a study of 255 patients undergoing primary long-term left ventricular assist device placement, patients with MELD or MELD-XI <17 had improved on-device and overall survival ($p < 0.05$) with a higher predictive power for MELD-XI. The patients who demonstrated improvement in MELD-XI score during device support had similar outcomes to those without liver dysfunction (Yang et al. 2012). Kim et al. (2013) studied the effect of MELD, MELDNa, and MELD-XI in ambulatory patients with hepatic dysfunction being evaluated for heart transplant and found that increased MELD and MELDNa had an increased independent risk of transplant.

MELD Limitations

Since the implementation of the MELD score, it has proven to be a more equitable method for organ allocation. However, there are certain conditions in which the

MELD score does not adequately predict the wait list mortality or prognosis. This has led to the development of the MELD exception.

MELD and Hepatocellular Carcinoma

Patients with HCC typically have cirrhosis but may not have advanced disease (and therefore a low MELD score) and yet are still at risk for death. The seminal study on LT in patients with HCC came out of Milan, Italy, and demonstrated that LT in patients with early HCC (one lesion <5 cm or three lesions each <3 cm) had comparable survival to patients without HCC, known as the Milan criteria (MC) (Mazzaferro et al. 1996). With the introduction of MELD, it was apparent that to enable patients with HCC and a low MELD score to have a reasonable chance of getting transplanted, they would need an exception to their biological MELD score. To try and equate the risk of dying with HCC with a biological MELD score, it was initially decided to allocate a MELD score of 24 for T1 lesions (<2 cm) and 29 for T2 (>2 cm and <5 cm, or three lesions <3 cm). This improved the probability of LT for patients with HCC on the waiting list (Yao et al. 2004). Comparing pre-MELD and post-MELD era patients with HCC listed for LT, investigators noted that the 5-month waiting list survival was 90.3% pre-MELD and 95.7% post-MELD ($P < 0.001$), demonstrating that the MELD exception benefited the HCC patients (Sharma et al. 2004).

The initial MELD exception point allocation was decreased to 24 in April 2003 and 22 in January 2005 for T2 lesions with a 10% increase in risk reflected in a rise in MELD score every 3 months. Both of these decreases occurred as it was noted that the allocated MELD score did not correlate to the risk of wait list dropout for these candidates. There have however been no further changes in the allocation of incremental exception points over time. The current policy states that an HCC candidate with tumor within MC may receive an exception MELD score, "equivalent to a 15% probability of candidate death within 3 months," with additional points given every 3 months, "equivalent to a 10% increase in candidate mortality." However, several studies have suggested this MELD exception is not such an accurate biomarker of poor outcome and may favor HCC patients (Massie et al. 2011; Washburn et al. 2010) and neither the initially nor the incrementally awarded MELD exception points for HCC accurately reflect the risk of wait list removal for HCC candidates, particularly when compared to non-HCC candidates (Goldberg et al. 2012). Currently, UNOS/OPTN is reviewing new changes to MELD exceptions such as capping HCC MELD to 34 points and to delay listing for HCC by 6 months.

MELD Exceptions for Other Conditions

MELD exceptions are also given for other conditions associated with ESLD that confer a risk of death but are not accurately measured by the MELD score (Freeman et al 2006; Table 3). These include pulmonary complications of cirrhosis,

Table 3 Conditions where a MELD exception is permitted on the LT waiting list

Condition	MELD exception points	Increase in points
Hepatocellular carcinoma (T2)	22	10% increase every 3 months
Familial amyloidosis polyneuropathy	22	10% increase every 3 months
Hepatopulmonary syndrome (HPS)	22	10% increase every 3 months
Portopulmonary hypertension	22	10% increase every 3 months
Cholangiocarcinoma (meeting protocol)	22	10% increase every 3 months
Cystic fibrosis	22	
Primary hyperoxaluria	28	
Metabolic disorders	30	If no transplant in 30 days, then status 1b

hepatopulmonary syndrome (characterized by PaO_2 <60 mmHg on room air) and portopulmonary hypertension (characterized by a mean pulmonary artery pressure ≥35 mmHg at diagnosis that must be maintained at <35 mmHg with treatment), and complications of liver disorders like primary sclerosing cholangitis leading to recurrent cholangitis. Patients with rare conditions like cystic fibrosis, familial amyloid polyneuropathy, polycystic liver disease, and primary oxaluria whose liver function is usually preserved but need a liver transplant also benefit from MELD exceptions. Patients listed with the MELD exception typically receive a 10% increase in their MELD score every 3 months while on the waiting list. In certain regions, centers can appeal for extra MELD points if the natural MELD of the patient does not reflect the underlying severity of liver disorder.

MELD and Posttransplant Outcome

The success of MELD for liver allocation and prognosis of other liver disorders has led to multiple studies evaluating the effect of the MELD score on posttransplant outcomes. Most have found no association between patient or graft outcome (Santori et al. 2005; Cywinski et al. 2011; Hayashi et al. 2003) although a single study suggested that MELD >25 was associated with poor patient and graft survival (Habib et al. 2006) and a MELD >23 predicts longer intensive care stay (Oberkofler et al. 2010). However, a MELD score >19 on postoperative day 5 may predict early graft dysfunction (Wagener et al. 2013).

Summary Points

1. The MELD score is an accurate biomarker of 90-day mortality in patients with ESLD, essentially measuring how sick a patient is.

2. Since 2002, the MELD score has been used to prioritize deceased donor organ allocation for LT in the USA.
3. The use of the MELD allocation system has resulted in sicker patients being transplanted with decreased waiting time, thereby decreasing the death rate on the LT waiting list, without an adverse effect on posttransplant outcome.
4. The MELD score has been adopted as a biomarker in other conditions associated with liver disease with good effect.
5. There are modifications of the MELD score that may have increased effectiveness in certain situations.
6. The MELD score is not an accurate biomarker for the risk of death from liver cancer and some other conditions and hence for the purposes of liver allocation on the transplant list a MELD exception can be given.

References

Ahmad J, Downey KK, Akoad M, Cacciarelli TV. Impact of the MELD score on waiting time and disease severity in liver transplantation in United States veterans. Liver Transpl. 2007a; 13(11):1564–9.

Ahmad J, Bryce CL, Cacciarelli T, Roberts MS. Differences in access to liver transplantation: disease severity, waiting time, and transplantation center volume. Ann Intern Med. 2007b; 146(10):707–13.

Ailawadi G, LaPar DJ, Swenson BR, Siefert SA, Lau C, Kern JA, Peeler BB, Littlewood KE, Kron IL. Model for end-stage liver disease predicts mortality for tricuspid valve surgery. Ann Thorac Surg. 2009;87(5):1460–8.

Alessandria C, Ozdogan O, Guevara M, Restuccia T, Jimenez W, Arroyo V, Rodes J, Gines P. MELD score and clinical type predict prognosis in hepatorenal syndrome: relevance to liver transplantation. Hepatology. 2005;41(6):1282–9.

Aranha GV, Sontag SJ, Greenlee HB. Cholecystectomy in cirrhotic patients: a formidable operation. Am J Surg. 1982;143(1):55–60.

Arroyo V, Gines P, Gerbes AL, Dudley FJ, Gentilini P, Laffi G, Reynolds TB, Ring-Larsen H, Scholmerich J. Definition and diagnostic criteria of refractory ascites and hepatorenal syndrome in cirrhosis. International Ascites Club. Hepatology. 1996;23(1):164–76.

Benckert C, Quante M, Thelen A, Bartels M, Laudi S, Berg T, Kaisers U, Jonas S. Impact of the MELD allocation after its implementation in liver transplantation. Scand J Gastroenterol. 2011;46(7–8):941–8.

Biggins SW, Rodriguez HJ, Bacchetti P, Bass NM, Roberts JP, Terrault NA. Serum sodium predicts mortality in patients listed for liver transplantation. Hepatology. 2005;41(1):32–9.

Biggins SW, Kim WR, Terrault NA, Saab S, Balan V, Schiano T, Benson J, Therneau T, Kremers W, Wiesner R, Kamath P, Klintmalm G. Evidence-based incorporation of serum sodium concentration into MELD. Gastroenterology. 2006;130(6):1652–60.

Bittermann T, Makar G, Goldberg D. Exception point applications for 15 points: an unintended consequence of the share 15 policy. Liver Transpl. 2012;18(11):1302–9.

Botta F, Giannini E, Romagnoli P, Fasoli A, Malfatti F, Chiarbonello B, Testa E, Risso D, Colla G, Testa R. MELD scoring system is useful for predicting prognosis in patients with liver cirrhosis and is correlated with residual liver function: a European study. Gut. 2003;52(1):134–9.

Chalasani N, Kahi C, Francois F, Pinto A, Marathe A, Bini EJ, Pandya P, Sitaraman S, Shen J. Model for end-stage liver disease (MELD) for predicting mortality in patients with acute variceal bleeding. Hepatology. 2002;35(5):1282–4.

Chawla YK, Kashinath RC, Duseja A, Dhiman RK. Predicting mortality across a broad spectrum of liver disease – an assessment of Model for End-Stage Liver Disease (MELD), Child–Turcotte–Pugh (CTP), and creatinine-modified CTP scores. J Clin Exp Hepatol. 2011;1(3):161–8.

Child CG, Turcotte JG. Surgery and portal hypertension. Major Probl Clin Surg. 1964;1:1–85.

Cho HC, Jung HY, Sinn DH, Choi MS, Koh KC, Paik SW, Yoo BC, Kim SW, Lee JH. Mortality after surgery in patients with liver cirrhosis: comparison of Child-Turcotte-Pugh, MELD and MELDNa score. Eur J Gastroenterol Hepatol. 2011;23(1):51–9.

Cholongitas E, Senzolo M, Patch D, Kwong K, Nikolopoulou V, Leandro G, Shaw S, Burroughs AK. Risk factors, sequential organ failure assessment and model for end-stage liver disease scores for predicting short term mortality in cirrhotic patients admitted to intensive care unit. Aliment Pharmacol Ther. 2006;23(7):883–93.

Cholongitas E, Marelli L, Kerry A, Goodier DW, Nair D, Thomas M, Patch D, Burroughs AK. Female liver transplant recipients with the same GFR as male recipients have lower MELD scores – ca systematic bias. Am J Transplant. 2007a;7(3):685–92.

Cholongitas E, Marelli L, Kerry A, Senzolo M, Goodier DW, Nair D, Thomas M, Patch D, Burroughs AK. Different methods of creatinine measurement significantly affect MELD scores. Liver Transpl. 2007b;13(4):523–9.

Cucchetti A, Ercolani G, Vivarelli M, Cescon M, Ravaioli M, La Barba G, Zanello M, Grazi GL, Pinna AD. Impact of model for end-stage liver disease (MELD) score on prognosis after hepatectomy for hepatocellular carcinoma on cirrhosis. Liver Transpl. 2006;12(6):966–71.

Cywinski JB, Mascha EJ, You J, Sessler DI, Kapural L, Argalious M, Parker BM. Pre-transplant MELD and sodium MELD scores are poor predictors of graft failure and mortality after liver transplantation. Hep Intl. 2011;5(3):841–9.

Dawwas MF, Lewsey JD, Neuberger JM, Gimson AE. The impact of serum sodium concentration on mortality after liver transplantation: a cohort multicenter study. Liver Transpl. 2007; 13(8):1115–24.

Dhiman RK, Jain S, Maheshwari U, Bhalla A, Sharma N, Ahluwalia J, Duseja A, Chawla Y. Early indicators of prognosis in fulminant hepatic failure: an assessment of the Model for End-Stage Liver Disease (MELD) and King's College Hospital criteria. Liver Transpl. 2007;13(6):814–21.

Dienstag JL, Ghany MG, Morgan TR, Di Bisceglie AM, Bonkovsky HL, Kim HY, Seeff LB, Szabo G, Wright EC, Sterling RK, Everson GT, Lindsay KL, Lee WM, Lok AS, Morishima C, Stoddard AM, Everhart JE; HALT-C Trial Group. A prospective study of the rate of progression in compensated, histologically advanced chronic hepatitis C. Hepatology. 2011;54(2):396–405. doi:10.1002/hep.24370. Epub 2011 Jun 23.

Dultz G, Seelhof M, Herrmann E, Welker MW, Friedrich-Rust M, Teuber G, Kronenberger B, von Wagner M, Vermehren J, Sarrazin C, Zeuzem S, Hofmann WP. Baseline MELD score predicts hepatic decompensation during antiviral therapy in patients with chronic hepatitis C and advanced cirrhosis. PLoS One. 2013;8(8):e71262.

Dunn W, Jamil LH, Brown LS, Wiesner RH, Kim WR, Menon KV, Malinchoc M, Kamath PS, Shah V. MELD accurately predicts mortality in patients with alcoholic hepatitis. Hepatology. 2005; 41(2):353–8.

Dutkowski P, De Rougemont O, Mullhaupt B, Clavien PA. Current and future trends in liver transplantation in Europe. Gastroenterology. 2010;138(3):802–9. e801–4.

Dutkowski P, Oberkofler CE, Bechir M, Mullhaupt B, Geier A, Raptis DA, Clavien PA. The model for end-stage liver disease allocation system for liver transplantation saves lives, but increases morbidity and cost: a prospective outcome analysis. Liver Transpl. 2011;17(6):674–84.

Fattovich G, Giustina G, Degos F, Tremolada F, Diodati G, Almasio P, Nevens F, Solinas A, Mura D, Brouwer JT, Thomas H, Njapoum C, Casarin C, Bonetti P, Fuschi P, Basho J, Tocco A, Bhalla A, Galassini R, Noventa F, Schalm SW, Realdi G. Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. Gastroenterology. 1997;112(2):463–72.

Freeman Jr RB, Edwards EB. Liver transplant waiting time does not correlate with waiting list mortality: implications for liver allocation policy. Liver Transpl. 2000;6(5):543–52.

Freeman RB, Wiesner RH, Edwards E, Harper A, Merion R, Wolfe R, P. United Network for Organ Sharing Organ, L. Transplantation Network, C. Transplantation. Results of the first year of the new liver allocation plan. Liver Transpl. 2004;10(1):7–15.

Freeman Jr RB, Gish RG, Harper A, Davis GL, Vierling J, Lieblein L, Klintmalm G, Blazek J, Hunter R, Punch J. Model for end-stage liver disease (MELD) exception guidelines: results and recommendations from the MELD Exception Study Group and Conference (MESSAGE) for the approval of patients who need liver transplantation with diseases not considered by the standard MELD formula. Liver Transpl. 2006;12(12 Suppl 3):S128–36.

Freitas AC, Itikawa WM, Kurogi AS, Stadnik LG, Parolin MB, Coelho JC. The impact of the model for end-stage liver disease (MELD) on liver transplantation in one center in Brazil. Arq Gastroenterol. 2010;47(3):233–7.

Garcia-Tsao G, Sanyal AJ, Grace ND, Carey W. Prevention and management of gastroesophageal varices and variceal hemorrhage in cirrhosis. Hepatology. 2007;46(3):922–38.

Goldberg D, French B, Abt P, Feng S, Cameron AM. Increasing disparity in waitlist mortality rates with increased model for end-stage liver disease scores for candidates with hepatocellular carcinoma versus candidates without hepatocellular carcinoma. Liver Transpl. 2012; 18(4):434–43.

Goyal SK, Dixit VK, Jain AK, Mohapatra PK, Ghosh JK. Assessment of the Model for End-stage Liver Disease (MELD) score in predicting prognosis of patients with alcoholic hepatitis. J Clin Exp Hepatol. 2014;4(1):19–24.

Habib S, Berk B, Chang CC, Demetris AJ, Fontes P, Dvorchik I, Eghtesad B, Marcos A, Shakil AO. MELD and prediction of post-liver transplantation survival. Liver Transpl. 2006; 12(3):440–7.

Hayashi PH, Forman L, Steinberg T, Bak T, Wachs M, Kugelmas M, Everson GT, Kam I, Trotter JF. Model for End-Stage Liver Disease score does not predict patient or graft survival in living donor liver transplant recipients. Liver Transpl. 2003;9(7):737–40.

Heuman DM, Abou-Assi SG, Habib A, Williams LM, Stravitz RT, Sanyal AJ, Fisher RA, Mihas AA. Persistent ascites and low serum sodium identify patients with cirrhosis and low MELD scores who are at high risk for early death. Hepatology. 2004;40(4):802–10.

Heuman DM, Mihas AA, Habib A, Gilles HS, Stravitz RT, Sanyal AJ, Fisher RA. MELD-XI: a rational approach to "sickest first" liver transplantation in cirrhotic patients requiring anticoagulant therapy. Liver Transpl. 2007;13(1):30–7.

Hsu KY, Chau GY, Lui WY, Tsay SH, King KL, Wu CW. Predicting morbidity and mortality after hepatic resection in patients with hepatocellular carcinoma: the role of Model for End-Stage Liver Disease score. World J Surg. 2009;33(11):2412–9.

Hsu CY, Lin HC, Huang YH, Su CW, Lee FY, Huo TI, Lee PC, Lee JY, Lee SD. Comparison of the model for end-stage liver disease (MELD), MELD-Na and MELDNa for outcome prediction in patients with acute decompensated hepatitis. Dig Liver Dis. 2010;42(2):137–42.

Huo TI, Wu JC, Lin HC, Lee FY, Hou MC, Lee PC, Chang FY, Lee SD. Evaluation of the increase in model for end-stage liver disease (DeltaMELD) score over time as a prognostic predictor in patients with advanced cirrhosis: risk factor analysis and comparison with initial MELD and Child-Turcotte-Pugh score. J Hepatol. 2005;42(6):826–32.

Huo TI, Lin HC, Hsia CY, Huang YH, Wu JC, Chiang JH, Chiou YY, Lui WY, Lee PC, Lee SD. The MELD-Na is an independent short- and long-term prognostic predictor for hepatocellular carcinoma: a prospective survey. Dig Liver Dis. 2008;40(11):882–9.

Jiang M. Comparison of four models for end-stage liver disease in evaluating the prognosis of cirrhosis. World J Gastroenterol. 2008;14(42):6546.

Kaiser T, Kinny-Koster B, Bartels M, Parthaune T, Schmidt M, Thiery J. Impact of different creatinine measurement methods on liver transplant allocation. PLoS One. 2014;9(2): e90015.

Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, Dickson ER, Kim WR. A model to predict survival in patients with end-stage liver disease. Hepatology. 2001;33(2):464–70.

Kim WR, Biggins SW, Kremers WK, Wiesner RH, Kamath PS, Benson JT, Edwards E, Therneau TM. Hyponatremia and mortality among patients on the liver-transplant waiting list. N Engl J Med. 2008;359(10):1018–26.

Kim MS, Kato TS, Farr M, Wu C, Givens RC, Collado E, Mancini DM, Schulze PC. Hepatic dysfunction in ambulatory patients with heart failure: application of the MELD scoring system for outcome prediction. J Am Coll Cardiol. 2013;61(22):2253–61.

Kremers WK, van IJperen M, Kim WR, Freeman RB, Harper AM, Kamath PS, Wiesner RH. MELD score as a predictor of pretransplant and posttransplant survival in OPTN/UNOS status 1 patients. Hepatology. 2004;39(3):764–9.

Luca A, Angermayr B, Bertolini G, Koenig F, Vizzini G, Ploner M, Peck-Radosavljevic M, Gridelli B, Bosch J. An integrated MELD model including serum sodium and age improves the prediction of early mortality in patients with cirrhosis. Liver Transpl. 2007;13(8):1174–80.

Lucey MR, Brown KA, Everson GT, Fung JJ, Gish R, Keeffe EB, Kneteman NM, Lake JR, Martin P, McDiarmid SV, Rakela J, Shiffman ML, So SK, Wiesner RH. Minimal criteria for placement of adults on the liver transplant waiting list: a report of a national conference organized by the American Society of Transplant Physicians and the American Association for the Study of Liver Diseases. Liver Transpl Surg. 1997 Nov;3(6):628–37.

Maddrey WC, Boitnott JK, Bedine MS, Weber Jr FL, Mezey E, White Jr RI. Corticosteroid therapy of alcoholic hepatitis. Gastroenterology. 1978;75(2):193–9.

Malinchoc M, Kamath PS, Gordon FD, Peine CJ, Rank J, ter Borg PC. A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts. Hepatology. 2000;31(4):864–71.

Massie AB, Caffo B, Gentry SE, Hall EC, Axelrod DA, Lentine KL, Schnitzler MA, Gheorghian A, Salvalaggio PR, Segev DL. MELD exceptions and rates of waiting list outcomes. Am J Transplant. 2011;11(11):2362–71.

Massie AB, Chow EKH, Wickliffe CE, Luo X, Gentry SE, Mulligan DC, Segev DL. Early changes in liver distribution following implementation of share 35. Am J Transplant. 2015; 15(3):659–67.

Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med. 1996;334(11):693–9.

Menon KV, Gores GJ, Shah VH. Pathogenesis, diagnosis, and treatment of alcoholic liver disease. Mayo Clin Proc. 2001;76(10):1021–9.

Merion RM, Schaubel DE, Dykstra DM, Freeman RB, Port FK, Wolfe RA. The survival benefit of liver transplantation. Am J Transplant. 2005;5(2):307–13.

Northup PG, Wanamaker RC, Lee VD, Adams RB, Berg CL. Model for End-Stage Liver Disease (MELD) predicts nontransplant surgical mortality in patients with cirrhosis. Ann Surg. 2005;242(2):244–51.

Oberkofler CE, Dutkowski P, Stocker R, Schuepbach RA, Stover JF, Clavien PA, Bechir M. Model of end stage liver disease (MELD) score greater than 23 predicts length of stay in the ICU but not mortality in liver transplant recipients. Crit Care. 2010;14(3):R117.

Organ Procurement and Transplantation Network. Health Resources and Services Administration, HHS. Final rule. [No authors listed]. Fed Regist. 1999 Oct 20;64(202):56650-61

Ostapowicz G, Lee WM. Acute hepatic failure: a western perspective. J Gastroenterol Hepatol. 2000;15(5):480–8.

Papatheodoridis GV, Cholongitas E, Dimitriadou E, Touloumi G, Sevastianos V, Archimandritis AJ. MELD vs Child-Pugh and creatinine-modified Child-Pugh score for predicting survival in patients with decompensated cirrhosis. World J Gastroenterol. 2005;11(20):3099–104.

Pomfret EA, Fryer JP, Sima CS, Lake JR, Merion RM. Liver and intestine transplantation in the United States, 1996–2005. Am J Transplant. 2007;7(5 Pt 2):1376–89.

Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. Br J Surg. 1973;60(8):646–9.

Quante M, Benckert C, Thelen A, Jonas S. Experience since MELD implementation: how does the new system deliver? Int J Hepatol. 2012;2012:264015.

Ruf AE, Kremers WK, Chavez LL, Descalzi VI, Podesta LG, Villamil FG. Addition of serum sodium into the MELD score predicts waiting list mortality better than MELD alone. Liver Transpl. 2005;11(3):336–43.

Said A, Williams J, Holden J, Remington P, Gangnon R, Musat A, Lucey MR. Model for end stage liver disease score predicts mortality across a broad spectrum of liver disease. J Hepatol. 2004;40(6):897–903.

Santori G, Andorno E, Morelli N, Antonucci A, Bottino G, Mondello R, Castiglione AG, Valente R, Ravazzoni F, Di Domenico S, Valente U. MELD score versus conventional UNOS status in predicting short-term mortality after liver transplantation. Transpl Int. 2005;18(1):65–72.

Schmidt LE, Larsen FS. MELD score as a predictor of liver failure and death in patients with acetaminophen-induced liver injury. Hepatology. 2007;45(3):789–96.

Schouten JN, Francque S, Van Vlierberghe H, Colle I, Nevens F, Delwaide J, Adler M, Starkel P, Ysebaert D, Gadisseur A, De Winter B, Smits JM, Rahmel A, Michielsen P. The influence of laboratory-induced MELD score differences on liver allocation: more reality than myth. Clin Transplant. 2012;26(1):E62–70.

Sharma P, Balan V, Hernandez JL, Harper AM, Edwards EB, Rodriguez-Luna H, Byrne T, Vargas HE, Mulligan D, Rakela J, Wiesner RH. Liver transplantation for hepatocellular carcinoma: the MELD impact. Liver Transpl. 2004;10(1):36–41.

Sharma P, Schaubel DE, Gong Q, Guidinger M, Merion RM. End-stage liver disease candidates at the highest model for end-stage liver disease scores have higher wait-list mortality than status-1A candidates. Hepatology. 2012;55(1):192–8.

Srikureja W, Kyulo NL, Runyon BA, Hu KQ. MELD score is a better prognostic model than Child-Turcotte-Pugh score or discriminant function score in patients with alcoholic hepatitis. J Hepatol. 2005;42(5):700–6.

Teh SH, Nagorney DM, Stevens SR, Offord KP, Therneau TM, Plevak DJ, Talwalkar JA, Kim WR, Kamath PS. Risk factors for mortality after surgery in patients with cirrhosis. Gastroenterology. 2007;132(4):1261–9.

Toshikuni N, Izumi A, Nishino K, Inada N, Sakanoue R, Yamato R, Suehiro M, Kawanaka M, Yamada G. Comparison of outcomes between patients with alcoholic cirrhosis and those with hepatitis C virus-related cirrhosis. J Gastroenterol Hepatol. 2009;24(7):1276–83.

Trotter JF, Brimhall B, Arjal R, Phillips C. Specific laboratory methodologies achieve higher model for end-stage liver disease (MELD) scores for patients listed for liver transplantation. Liver Transpl. 2004;10(8):995–1000.

Trotter JF, Olson J, Lefkowitz J, Smith AD, Arjal R, Kenison J. Changes in international normalized ratio (INR) and model for end-stage liver disease (MELD) based on selection of clinical laboratory. Am J Transplant. 2007;7(6):1624–8.

van Deursen VM, Damman K, Hillege HL, van Beek AP, van Veldhuisen DJ, Voors AA. Abnormal liver function in relation to hemodynamic profile in heart failure patients. J Card Fail. 2010; 16(1):84–90.

Villanueva C, Piqueras M, Aracil C, Gomez C, Lopez-Balaguer JM, Gonzalez B, Gallego A, Torras X, Soriano G, Sainz S, Benito S, Balanzo J. A randomized controlled trial comparing ligation and sclerotherapy as emergency endoscopic treatment added to somatostatin in acute variceal bleeding. J Hepatol. 2006;45(4):560–7.

Wagener G, Raffel B, Young AT, Minhaz M, Emond J. Predicting early allograft failure and mortality after liver transplantation: the role of the postoperative model for end-stage liver disease score. Liver Transpl. 2013;19(5):534–42.

Washburn K, Edwards E, Harper A, Freeman R. Hepatocellular carcinoma patients are advantaged in the current liver transplant allocation system. Am J Transplant. 2010;10(7):1643–8.

Wiesner R, Edwards E, Freeman R, Harper A, Kim R, Kamath P, Kremers W, Lake J, Howard T, Merion RM, Wolfe RA, Krom R, C. United Network for Organ Sharing Liver Disease Severity

Score. Model for end-stage liver disease (MELD) and allocation of donor livers. Gastroenterology. 2003;124(1):91–6.
Yang JA, Kato TS, Shulman BP, Takayama H, Farr M, Jorde UP, Mancini DM, Naka Y, Schulze PC. Liver dysfunction as a predictor of outcomes in patients with advanced heart failure requiring ventricular assist device support: use of the Model of End-stage Liver Disease (MELD) and MELD eXcluding INR (MELD-XI) scoring system. J Heart Lung Transplant. 2012;31(6):601–10.
Yantorno SE, Kremers WK, Ruf AE, Trentadue JJ, Podesta LG, Villamil FG. MELD is superior to King's college and Clichy's criteria to assess prognosis in fulminant hepatic failure. Liver Transpl. 2007;13(6):822–8.
Yao FY, Bass NM, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: lessons from the first year under the Model of End-Stage Liver Disease (MELD) organ allocation policy. Liver Transpl. 2004;10(5):621–30.
Ziser A, Plevak DJ, Wiesner RH, Rakela J, Offord KP, Brown DL. Morbidity and mortality in cirrhotic patients undergoing anesthesia and surgery. Anesthesiology. 1999;90(1):42–53.

Mechanistic Biomarkers in Liver Diseases

4

Mitchell R. McGill, Benjamin L. Woolbright, James L. Weemhoff, and Hartmut Jaeschke

Contents

M.R. McGill (✉)
Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA

Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA
e-mail: mmcgill@kumc.edu; mmcgill@path.wustl.edu

B.L. Woolbright • J.L. Weemhoff • H. Jaeschke
Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA
e-mail: bwoolbright@kumc.edu; jweemhoff@kumc.edu; hjaeschke@kumc.edu

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_5

Abstract

Several biomarkers are used in the diagnosis and monitoring of liver injury and liver disease. However, these markers have major limitations, including poor correlation with histology in some diseases and lack of prognostic utility. Recently, a number of new serum and urine biomarkers have been developed to enable the study of fundamental molecular mechanisms of tissue injury. These markers can provide information about the mode of cell death, mitochondrial and nuclear damage, oxidative stress, and inflammation. Importantly, several of these have also been shown to have clinical utility in early studies of liver disease patients. The purpose of this chapter is to provide an overview of the major groups of mechanistic biomarkers that have recently been applied to the study of liver injury and to discuss their potential use in the clinic and in the study of disease in other organs.

Keywords

Cell death • Mitochondria • Inflammation • Oxidative stress • Hepatotoxicity

List of Abbreviations

ALD	Alcoholic liver disease
APAP	Acetaminophen
BDL	Bile duct ligation
DAMP	Damage-associated molecular pattern
DILI	Drug-induced liver injury
ERCP	Endoscopic retrograde cholangiopancreatography
ITBL	Ischemic-type biliary lesion
K18	Keratin 18
miRNA	MicroRNA
mtDNA	Mitochondrial DNA
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
OLT	Orthotopic liver transplant

Key Facts of Mechanistic Biomarkers in Acetaminophen Toxicity

- Biomarkers of mitochondrial damage and nuclear DNA fragmentation are elevated in acetaminophen hepatotoxicity.
- Biomarkers of inflammation are elevated in acetaminophen hepatotoxicity.

- Cell death biomarkers confirm that the primary mode of cell death in acetaminophen hepatotoxicity is oncotic necrosis.
- Mechanistic biomarkers predict poor prognosis in acetaminophen overdose patients with acute liver failure.

Key Facts of Mechanistic Biomarkers in Ischemia-Reperfusion Injury

- Circulating high-mobility group box 1 (HMGB1) increases in IRI.
- Acetylated HMGB1 increases late in IRI, indicating late inflammation.
- The cell death biomarker K18 has revealed that the primary mode of cell death in IRI is oncotic necrosis.
- miR-122 increases in circulation during IRI.

Key Facts of Mechanistic Biomarkers in Chronic Liver Disease

- Cell death biomarkers are elevated in multiple chronic liver diseases.
- miR-122 is elevated in serum in many chronic liver diseases.
- Acetylated HMGB1 and cytokines increase and suggest that inflammation occurs in some chronic liver diseases.
- Keratin 18 data suggest that apoptosis likely plays a role in some chronic liver diseases.

Definition of Words and Terms

Acute liver failure	Failure of the liver characterized by impaired synthetic function (INR >1.5) and encephalopathy. Can be hyperacute (develops in <7 days), acute (7–21 days), or subacute (21–26 days).
Acute liver injury	Liver injury that develops and resolves quickly in the absence of a pre-existing liver disease.
Apoptosis	One of the two most widely recognized modes of cell death. Characterized by protease-mediated destruction of cells, with cell shrinkage, nuclear chromatin condensation, membrane blebbing, and formation of apoptotic bodies.
Biomarker	Biochemical indicator (usually an endogenous small molecule, protein, or nucleic acid) that can be used for diagnosis or prognosis. Can be measured in one or more biological fluids (serum, urine, saliva, or CSF) or tissues. Noninvasive biomarkers are generally measured in fluids.

Cholestasis	Reduced or obstructed flow of bile acids in the hepatobiliary system. Can be extrahepatic (e.g., obstruction of the common bile duct) or intrahepatic (e.g., reduced secretion of bile acids from hepatocytes).
Chronic liver disease	Persistent disease of the liver. Usually accompanied by inflammation and results in fibrosis or, in advanced cases, cirrhosis. Often caused by viral hepatitis, alcoholic hepatitis, or nonalcoholic fatty liver disease.
Damage-associated molecular patterns	Endogenous molecules that can initiate and perpetuate inflammation through activation of pattern recognition receptors (e.g., Toll-like receptors) on innate immune cells.
Drug-induced liver injury	Liver injury (usually acute) caused by one or more drugs. Idiosyncratic DILI is rare (<1 in 10,000 drug exposures) and characterized by prolonged therapeutic exposure and likely involves an immune component. Intrinsic DILI is common, can occur after a single drug overdose, and exhibits a clear dose-response.
Ischemia-reperfusion	Obstruction of the blood supply to a tissue, followed by restoration of normal blood flow. Tissue becomes hypoxic during the ischemic phase, and the return of normal perfusion initiates multiple mechanisms of cell damage and death.
Mechanisms	The underlying molecular and biochemical events that lead to disease or injury.
Oncotic necrosis	One of the two most widely recognized modes of cell death. Characterized by cell swelling, membrane lysis, and nuclear DNA fragmentation and often caused or accompanied by mitochondrial damage and dysfunction.

Introduction

The most commonly used noninvasive clinical biomarkers of liver disease can be divided into two groups: markers of liver injury and markers of liver function. The aminotransferases alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are the best-known examples of liver injury biomarkers. The presence of these two enzymes in serum, particularly ALT, is generally thought to indicate loss of hepatocyte membrane integrity. This is supported by experiments with various models of apoptosis in mice: the initial apoptotic hepatocyte death, which does not

involve the destruction of cell membranes, can be observed by morphology, caspase activation, and DNA fragmentation, but causes little or no ALT release (Bajt et al. 2000). On the other hand, oncotic necrosis occurring at later time points, after the apoptosis, does result in massive ALT release (Bajt et al. 2000). Biomarkers of liver function include serum bilirubin and various measures of coagulation time. Bilirubin is a product of the breakdown of red blood cells. The compound is not water soluble, so unconjugated bilirubin released by hemolysis binds to serum albumin while in circulation. The bilirubin is then taken up by hepatocytes, where it is conjugated with glucuronide to improve solubility and excreted into bile for elimination in feces. High concentrations of unconjugated bilirubin usually suggest a hemolytic disease, while high concentrations of conjugated bilirubin usually suggest liver dysfunction and failure to properly excrete the compound. The liver also produces many of the coagulation factors normally found in blood, so liver damage or dysfunction can lead to coagulopathy.

Although these existing biomarkers are useful, it is clear that new biomarkers of liver disease are needed. Unfortunately, while the aminotransferases and liver function parameters are useful for diagnosis after the onset of disease, both have significant shortcomings. For example, neither works well for prognosis in cases of acute liver injury. It has been shown that ALT and AST generally do not correlate with outcome in drug-induced liver injury patients (Antoine et al. 2012; McGill et al. 2014c). Furthermore, although coagulation time (e.g., prothrombin time and INR) and bilirubin do correlate with outcome in some cases (O'Grady et al. 1989), they are generally elevated at late time points in the disease progression, when treatment decisions are already being made. Finally, in some patients with chronic liver disease (namely, viral hepatitis), markers such as ALT and AST may not be elevated above the normal range despite histological evidence of liver damage (Marcellin et al. 1999).

One promising new approach to the development of disease biomarkers is to search for indicators of pathophysiological mechanisms. These "mechanistic biomarkers" have several possible advantages (McGill and Jaeschke 2014). One could reasonably expect such a biomarker to appear in circulation early in the course of tissue injury because any mechanistic event that contributes to the injury must obviously occur before the injury itself. Of course, the former assumes there is some way for the target cell to release or export the marker into the extracellular space before cell death begins. In addition to traditional secretion pathways, emerging evidence shows that export of macromolecules into circulation can be accomplished by exosome release or microvesicle shedding, while release of low molecular weight compounds can occur through transporters. Another possible advantage of mechanistic biomarkers is improved prediction of patient outcome. For example, if a particular pathway is critical for progression of a given disease, then one might expect that patients with poor outcome would have greater activation of that pathway and thus possibly higher levels of some index of the pathway in their tissue or fluids.

This chapter summarizes major findings in mechanistic biomarker research in the hepatology field. Recent findings using examples of both acute and chronic liver

injury will be described based on data from both preclinical and clinical studies. Finally, we will discuss the potential future use of these biomarkers in the clinic for both liver and other diseases. It should be noted that, although there are some urine biomarkers that appear to be useful in liver disease, most research to date has focused on biomarkers in circulation.

Emerging Mechanistic Biomarkers

Markers of Cell Death

The two most widely studied modes of cell death are oncotic necrosis and apoptosis (Fadeel and Orrenius 2005). The former is characterized by cell swelling, nuclear disintegration, and plasma membrane rupture, while the latter is characterized by cell shrinkage, nuclear condensation, and membrane blebbing. The traditional apoptosis pathway involves activation of **c**ysteine-dependent **asp**artate-directed prote**ases** (caspases). When death receptors (e.g., Fas, tumor necrosis factor-α (TNF-α) receptor) in cell membranes are activated by their respective ligands (e.g., Fas ligand, TNF-α), the receptors oligomerize and recruit other proteins, e.g., Fas-associated death domain (FADD) protein, TNF receptor-associated death domain (TRADD) protein, and receptor-interacting protein (RIP) kinase 1 on the cytosolic side, that then initiate downstream signals within the cell (Fadeel and Orrenius 2005). When RIP1 is polyubiquitinated, it forms a platform for interaction with proteins that would otherwise inhibit nuclear factor k-light-chain-enhancer of activated B cells (NF-kB) (O'Donnell et al. 2007). This allows NF-kB to translocate into the nucleus and promote expression of pro-survival genes. At the same time, the proteins recruited to the death receptors can also activate caspase 8. In hepatocytes, caspase 8 cleaves Bid to form tBid, which translocates to mitochondria and forms a pore in the outer mitochondrial membrane. The outer mitochondrial membrane pore facilitates release of pro-apoptotic intermembrane proteins, including cytochrome c (Fadeel and Orrenius 2005). The latter forms a complex with the apoptotic protease-activating factor 1 (Apaf-1) and ATP, which activates caspase 9. Caspase 9 activates caspase 3 in turn, and caspase 3 is responsible for the execution of cell death by cleaving its numerous downstream targets (Fadeel and Orrenius 2005). This mitochondrial pathway serves to amplify the upstream apoptotic signal (Bajt et al. 2000). Because hepatocytes generally require the mitochondrial pathway for apoptosis execution, they are considered type II cells. However, under certain conditions when the apoptotic signal is strong enough to bypass the mitochondria, hepatocytes can also act as type I cells (Schüngel et al. 2009).

If there is enough cell damage or release of intracellular contents through some other mechanism, then the mode of cell death can be assessed by measuring markers of apoptosis in circulation. Several markers are available for this purpose (Fig. 1). The most direct way to assess apoptosis is to simply measure caspase 3 activation. This can be done in two ways: (1) immunoassays for the cleaved,

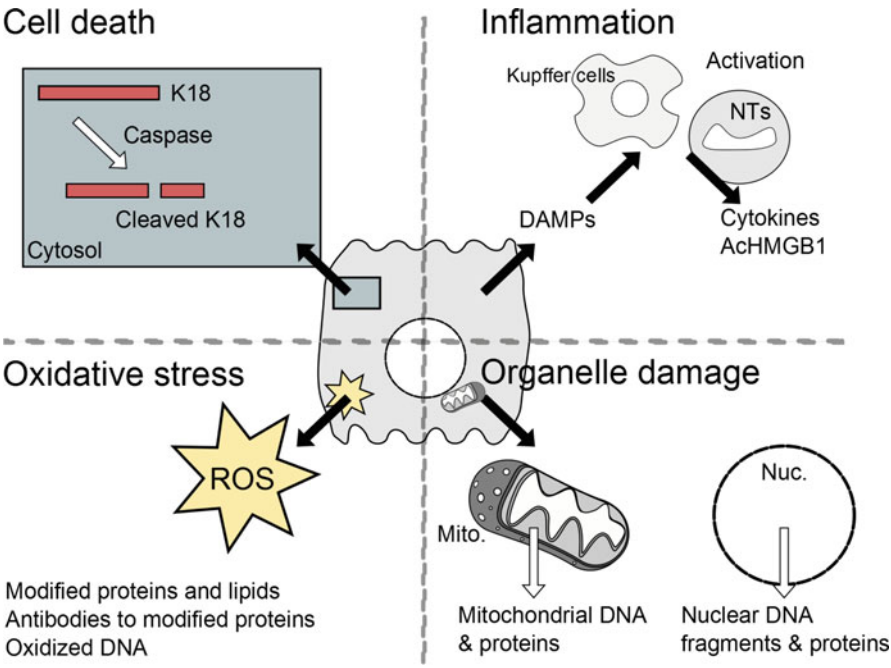

Fig. 1 Summary schematic of mechanistic biomarker release. Serum and urine biomarkers of cell death, inflammation, oxidative stress, and organelle damage have been described. Several examples are provided. Mode of cell death can be determined by measuring cleaved forms of caspase substrate proteins, including keratin 18 (K18). Inflammation can be detected by measuring immune cell activation, cytokine levels, acetylated HMGB1 (acHMGB1), and damage-associated molecular patterns (DAMPs). Oxidative stress can be assessed by measuring oxidized proteins, lipids, and DNA, as well as other markers, such as antibodies to oxidized proteins. Organelle damage can be measured based on release of organelle contents into the cytosol and subsequently into blood

active form of caspase 3 can be used in serum, and (2) kinetic assays of caspase 3 activity using fluorogenic substrates can be performed with serum samples. Both have been shown to work in mice treated with galactosamine/endotoxin (McGill et al. 2012), a well-established method for induction of TNF-mediated apoptosis in the liver. Another approach is to measure the cleavage products of activated caspases. Currently, the most popular example is the caspase-cleaved form of the structural protein keratin 18 (K18). Leers et al. (1999) characterized a monoclonal antibody, referred to as M30, which can bind to a specific epitope on K18 that is exposed by caspase cleavage. Another antibody, called M65, which recognizes both the cleaved and non-cleaved forms of K18, has also been developed, and it is generally thought that the ratio of M30-binding to M65-binding K18 can be calculated to obtain an approximation of the percentage of cell death occurring in the form of apoptosis in the course of a given disease (Ueno et al. 2005). However, K18 is not the only option. Theoretically, any caspase target could be measured. Although the full-length form of K18 is

sometimes said to be a specific biomarker for necrosis, the mechanism of K18 release has not been well studied and that idea is up for debate. The same is true for many intact proteins and other macromolecules that are released from dying cells – they are generally thought of as markers of oncotic necrosis, but the truth of this idea will depend upon the specific mechanism(s) of release for the macromolecules(s) in question.

Markers of Nuclear DNA Damage

Historically, internucleosomal nuclear DNA fragmentation was often considered a marker of apoptosis because caspases can activate specific DNases that cleave DNA into small pieces, while necrosis was thought to result in the formation of fewer, larger DNA fragments. However, internucleosomal DNA degradation can also occur in examples of oncotic necrosis (Bajt et al. 2006; Cover et al. 2005), which does not involve caspase activation. Thus, nuclear DNA fragmentation cannot be said to be specific for any mode of cell death (Fig. 1). Nevertheless, the measurement of nuclear DNA fragments is certainly one way to assess nuclear DNA damage and is currently the most common approach for that purpose. This can be accomplished in two major ways. First, DNA can be isolated from samples and run on an agarose gel with a DNA-binding dye to visualize the fragmentation. This approach is frequently used when working with tissue or cell culture samples (Duke et al. 1983; Cover et al. 2005). Similar techniques were applied to serum samples as early as the late 1980s (Boender et al. 1989), but more recently the use of immunoassays to measure DNA fragments in serum has become common (McGill et al. 2012, 2014c). These assays generally capture and immobilize nucleosomes using anti-histone antibodies and then detect the broken ends of the histone-bound DNA strands. Quantitative PCR for nuclear DNA is a third approach; however, although there is evidence that DNA copy number in serum by PCR correlates reasonably well with the anti-histone immunoassay methods (Holdenrieder et al. 2005), PCR methods are not specific for broken DNA.

Markers of Mitochondrial Damage

A method for assessment of dysfunction of mitochondrial components that is commonly used in newborn screening for metabolic disorders is the analytical measurement of acylcarnitines (Van Hove et al. 1993). Interestingly, there is considerable evidence that this approach can also be used to measure mitochondrial damage in liver injury (Chen et al. 2009; McGill et al. 2014b; Bhattacharyya et al. 2014). Acylcarnitines are conjugates of carnitine and acyl groups of fatty acids. Long-chain acylcarnitines are taken up by transporters in mitochondrial membranes, where the acyl groups are separated to undergo β-oxidation. When mitochondria are damaged or when one of the transporters or enzymes involved in

oxidation is impaired, acylcarnitines accumulate and become elevated in circulation.

Other biomarkers that are thought to indicate mitochondrial damage include several mitochondrial proteins and mitochondrial DNA (mtDNA). Examples of such proteins include the mitochondrial isoform of AST (Panteghini et al. 1984), cytochrome c (Miller et al. 2008), glutamate dehydrogenase (GLDH) (McGill et al. 2012), carbamoyl phosphate synthetase 1 (CPS-1) (Weerasinghe et al. 2014), and ATP synthase subunit-β (Whitaker et al. 2015). It is generally believed that mitochondrial matrix enzymes are liberated only when mitochondria are so severely damaged that mitochondrial membrane integrity is lost, and there is evidence that supports this idea (McGill et al. 2012). Similar evidence is available for mtDNA (McGill et al. 2012).

Markers of Oxidative Stress

Numerous approaches are available to measure oxidative stress using serum or plasma samples. Biomarkers of oxidative stress include nitrated and oxidized proteins (e.g., nitrotyrosine, protein carbonyls, oxidized albumin), oxidized DNA (Shigenaga et al. 1989), lipid peroxidation products (e.g., F2-isoprostanes, malondialdehyde), and circulating antibodies against oxidatively modified proteins (Rigamonti et al. 2003). Before beginning an experiment, it is critical to determine the source of oxidative stress to decide what marker would be most appropriate for a given study. For example, markers of lipid peroxidation generally would not be useful for the measurement of oxidative stress that occurs in the aqueous environment of the cytoplasm and vice versa.

Markers of Inflammation

Similar to oxidative stress, there are many ways to measure inflammation (Fig. 1). The most obvious approach is to measure pro-inflammatory cytokines, or the balance of pro- and anti-inflammatory cytokines, in circulation. For example, interleukins (IL-6, IL-8, etc.) have been measured as indicators of inflammation (James et al. 2005). When interpreting the results from these experiments, it is important to remember that circulating cytokine levels can substantially fluctuate over time and may also be affected by sampling procedure (Altara et al. 2015). Furthermore, there is evidence from rodents that not all cytokines increase in every case of inflammation (Moldawer et al. 1987). Thus, caution should be taken to avoid overinterpretation of circulating cytokine concentrations. Like cytokines, damage-associated molecular patterns (DAMPs) (e.g., high-mobility group box 1 (HMGB1) protein, mtDNA, N-formyl peptides, etc.) can also be measured in circulation (Antoine et al. 2012; Zhang et al. 2010; McGill et al. 2012, 2014c). Because DAMPs can initiate inflammatory responses by binding to Toll-like

receptors on inflammatory cells, they can be considered partial evidence for inflammation in various disease states.

Another approach is to measure cytokines or DAMPs with specific posttranslational modifications that indicate that they are being produced in response to a pro-inflammatory stimulus and are not simply passively released. For example, HMGB1 is normally present in nuclei. However, in inflammation, it is hyperacetylated in macrophages and other immune cells, and this causes it to instead be released into the extracellular space (Bonaldi et al. 2003). While non-acetylated HMGB1 is thought of as a DAMP because it is released from nuclei during cell death, hyperacetylated HMGB1 is a biomarker of the downstream inflammatory cell activation.

Finally, inflammation can be more directly assessed by measuring the abundance and/or activation of inflammatory cells in the circulation. For example, neutrophil number and priming or monocyte type can be assayed by flow cytometry (Williams et al. 2014; Antoniades et al. 2012). However, it is important to remember that sample collection and handling can dramatically affect the activation status of immune cells in blood.

Other Biomarkers

Various microRNAs (miRNAs) have recently emerged as very promising circulating biomarkers of liver injury and disease (Ward et al. 2014; McGill and Jaeschke 2015). miRNAs have also been measured in urine during liver injury (Yang et al. 2015). Although the mechanistic significance of most miRNAs in the liver is still being explored, it is thought that some are released as signaling mediators to be taken up by other cells (Royo and Falcon-Perez 2012; McGill and Jaeschke 2015). Thus, it is possible that specific miRNAs have mechanistic significance. For example, it has been suggested that exosome-bound miR-122 released from hepatocytes during alcohol exposure can signal to monocytes in the liver (Momen-Heravi et al. 2015). However, due to the large number of miRNAs expressed in mammalian tissues and their pleiotropic nature, extensive additional work is needed to understand all of the mechanistic implications of various changes in circulating miRNAs.

Bile acids can be measured as biomarkers of liver dysfunction. There is evidence that bile acid changes can either result from or initiate various signaling pathways leading to cell death or regeneration in the liver (Beger et al. 2015). Interestingly, not all bile acids necessarily increase together during liver injury; there can be differences in serum levels of specific bile acids, and these differences may shed light on mechanisms of liver injury (Woolbright et al. 2014b). However, further study may be needed to better understand the relationships between specific bile acids and pathophysiological mechanisms of liver diseases.

Finally, in the case of xenobiotic-induced liver injury, adducts of a drug or its metabolite(s) with macromolecules can often be measured as an indication of protein or DNA binding. Although such markers are typically thought of as indicators of exposure, covalent modification of macromolecules is a common and necessary first

step in the mechanisms of hepatotoxicity caused by many xenobiotics (McGill and Jaeschke 2014; Xie et al. 2015).

Mechanistic Biomarkers in Chronic Liver Diseases

Chronic liver disease is a major cause of morbidity and mortality, with a growing rate of incidence worldwide. Due to the inherent danger in performing liver biopsies on patients with chronic liver disease, considerable effort has gone toward defining prognostic, diagnostic, and mechanistic biomarkers that will prevent the need for invasive procedures. A number of these mechanistic biomarkers have been evaluated in a broad spectrum of liver diseases. As such, these biomarkers will be reviewed on the basis of liver disease etiology.

Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis

One of the first diseases with a push for the use of mechanistic biomarkers for the determination of mechanisms behind the pathophysiology was nonalcoholic fatty liver disease (NAFLD) or its inflammatory sequel, nonalcoholic steatohepatitis (NASH) (Fig. 2). Apoptosis was initially characterized as a major cause for hepatocyte cell death in NAFLD and NASH patients (Feldstein et al. 2003) and is likely mediated by activation of the c-Jun N-terminal kinase pathway (Singh et al. 2009). Subsequent studies both in vitro and in vivo have supported this hypothesis. Caspase 3 inhibition protects against diet-induced steatosis in some models of fatty liver (Thapaliya et al. 2014), and administration of free fatty acids causes apoptosis in hepatocytes, cholangiocytes, and cultured hepatoma lines (Cazanave et al. 2009). Initial measurements of serum cytokeratin-18 values (M30) demonstrated that not only was apoptosis positively correlated with progression from simple steatosis to steatohepatitis, but M30 values correlated strongly with tissue levels of apoptosis, indicating apoptosis was likely a major component of the injury process (Tamimi et al. 2011). M30 values have further been shown to be useful in staging of NAFLD before bariatric surgery (Diab et al. 2008), although larger studies indicated they do not perform well for staging NASH (Cusi et al. 2014). Elevated levels of the pro-apoptotic soluble Fas protein are also present in these patients, which may be indicative of Fas activation and Fas-mediated apoptosis in human patients (Feldstein et al. 2003; Tamimi et al. 2011). M65 levels have been measured in fewer studies, but are also elevated above control values (Yilmaz et al. 2007). The M30-to-M65 ratio indicated approximately 50% of cell death was through apoptosis, further characterizing potential benefit of targeting apoptosis pharmacologically in patients with NAFLD or NASH (Yilmaz et al. 2007).

As NASH is widely believed to be an inflammatory disorder (reviewed in Farrell et al. 2012), specific aspects of the inflammatory cascade may serve as important mechanistic biomarkers of injury. Markers of activated inflammatory cells correlate strongly with injury and progression in childhood NAFLD patients (De Vito

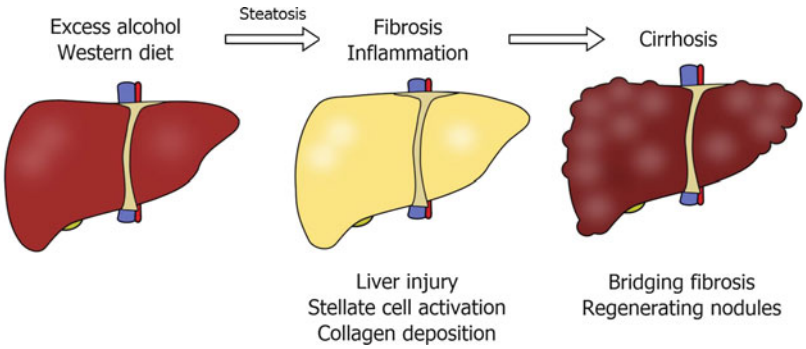

Fig. 2 Progression of chronic liver disease. Excess alcohol intake, high-fat diet, and other factors can lead to chronic conditions, such as steatosis, steatohepatitis, and fibrosis. Cirrhosis develops with chronic inflammation and bridging fibrosis and is characterized by patches of regenerating hepatocytes

et al. 2012). CXCL10 is protective against methionine-/choline-deficient diet-induced steatosis and also upregulated in human patients suggesting CXC chemokines may play a role in the recruitment of inflammatory cells such as neutrophils (Zhang et al. 2014). Soluble levels of the RAGE receptor, a receptor for advanced glycation end products such as HMGB1, are also present in serum of NAFLD patients and are inversely correlated with ALT, indicating activators of inflammation are likely responsible for a portion of the increase in injury (Yilmaz et al. 2007). Surprisingly, low levels of cathepsin D were strongly associated with progression to inflammation in childhood patients with NASH and also associated with higher ALT values and NAFLD activity score (Walenbergh et al. 2015). It is unknown how cathepsin D relates to NASH development, but implicates lysosomal dysfunction as a potential mediator of the shift from simple steatosis to advanced inflammation and disease. Further work in this area is necessary to better understand if mechanistic biomarkers can be applied to understand inflammation in NASH.

Alcoholic Hepatitis and Alcoholic Liver Disease

Alcoholic liver disease is a common problem in Western culture due to excessive consumption of alcohol. Chronic consumption of alcohol results in a spectrum of pathological states that are typically progressive from simple steatosis to inflammation, to fibrosis, and to cirrhosis and ending in hepatocellular carcinoma (Gao and Bataller 2011) (Fig. 2). On top of this spectrum of disease, some patients further deteriorate into an acute-on-chronic liver failure syndrome called alcoholic hepatitis (Gao and Bataller 2011). Due to the inability of mouse models to mimic human alcoholic liver disease, mechanistic biomarkers may be especially viable as a means of determining mechanisms of liver injury in humans and progression to more serious types of disease. Limited measurements of serum M30 and M65 have been

performed in large populations of specific populations of alcoholic patients; however, preliminary studies indicate elevations in both M30 and M65 in heavy drinking patients (Lavallard et al. 2011). Moreover, these values were independent risk factors for the development of fibrosis, and receiver operating characteristic (ROC) curves confirmed these assays could be predictive for fibrosis (Lavallard et al. 2011). miRNA release and dysregulation also occur during alcoholic hepatitis (McDaniel et al. 2014). miR-122 and miR-155 levels are increased in the exosome fraction of alcohol-treated mice (Bala et al. 2012). Increased levels of miR-155 likely contribute to TNF-α release in ALD mice, potentially through a protective mechanism involving miR-155 in the gut (Lippai et al. 2014), suggesting higher levels of miR-155 are pathogenic during ALD (Bala et al. 2011). Further work characterizing these markers in large population groups with biopsy-staged patients is warranted as cell death may be a major driver of clinical outcomes in both alcoholic hepatitis and alcoholic liver disease (Gao and Bataller 2011); the use of miRs and HMGB1 may be useful in better understanding this cell death.

Alcoholic hepatitis and alcoholic liver disease are also widely considered to involve inflammation as both a mechanism of disease progression and a mechanism of cell death associated with the disease (Gao and Bataller 2011). The inflammatory state associated is widely presumed to involve activation of TLR4 on macrophages by LPS; however, other TLR4 ligands are also increased in serum during alcoholic hepatitis in both mice and patients (Iracheta-Vellve et al. 2015). HMGB1 levels are elevated both in mice and in alcoholic patients in both the liver and serum (Ge et al. 2014). Interestingly, alcoholic patients and alcohol-fed mice were found to have both native HMGB1 and also hyperacetylated HMGB1 in the serum, suggesting not only was there cellular necrosis but likely activation of macrophages as well (Ge et al. 2014). Macrophage activation is a well-defined aspect of ALD pathogenesis in mice, which corroborates the use of HMGB1 and posttranslational modified versions of HMGB1 as mechanistic biomarkers in the field. Further investigation into the role of HMGB1 in human patients may be warranted.

Obstructive Cholestasis and Biliary Stricture

Cholestasis is a common pathological component of a variety of different disorders ranging from chronic autoimmune syndromes to acute obstruction of the bile ducts (Woolbright and Jaeschke 2012). A number of studies have demonstrated that administration of hydrophobic bile acids to rat hepatocytes results in a well-characterized apoptotic response (Malhi et al. 2010). This led to the widespread hypothesis that hepatocyte cell death and liver dysfunction during obstructive cholestasis were due to hepatocellular apoptosis (Malhi et al. 2010). Recent use of mechanistic biomarkers in both mouse models (Woolbright et al. 2013) and human patients (Woolbright et al. 2015) has challenged this hypothesis. BDL in the mouse results exclusively in the release of the M65 form of cytokeratin-18 into serum, significant increases in HMGB-1 and acetylated HMGB1, and increases in serum miR-122 levels (Woolbright et al. 2013), indicative of widespread hepatic necrosis in

this model. These data support previous studies in the BDL model that indicate necrosis and not apoptosis as the primary modality of injury (Gujral et al. 2004). Similar studies done in human patients suggest that obstructive cholestasis also results primarily in increases in M65 in human patients, as well as HMGB1 and acetylated HMGB1 release into serum (Woolbright et al. 2015). Interestingly, M30 values were also elevated in patients with obstructive cholestasis; however, this value was only ~10% of the M65 value, indicating apoptosis was a minor contribution of the overall injury. Increases in HMGB1 and acetylated HMGB1 support the idea that cholestasis results in a pro-inflammatory environment in both mice and human patients (Gujral et al. 2003; Woolbright et al. 2015). It has yet to be determined if this pro-inflammatory environment contributes directly in man as it does in mouse models to the actual injury (Woolbright and Jaeschke 2012). This considerable difference between the in vivo models and rat hepatocytes may be due to differences found in bile acid accumulation previously unrecognized (Zhang et al. 2012). Individual bile acid levels were measured in serum of mice and human patients where it was found that the majority of bile acids that accumulate in mice were not those known to cause toxicity (Zhang et al. 2012) and that while humans accumulate significantly more toxic, hydrophobic bile acids such as glycochenodeoxycholate (GCDC), values of GCDC consistent with toxicity caused necrosis and not apoptosis (Woolbright et al. 2015). These data parallel a study using administration of lithocholic acid in the mouse (Woolbright et al. 2014a). Lithocholic acid administration results in increases in metabolic derivatives of LCA including conjugate primary and secondary bile acids that can directly induce liver injury (Woolbright et al. 2014a). As individual bile acids have varied effects and varied levels of toxicity, measurements of individual bile acids can potentially be used to help determine mechanistic aspects of the pathophysiology. Disease states that result in significant accumulation of cytotoxic bile acids such as GCDC may be more likely to result in direct bile acid-induced necrosis than those that result primarily in accumulation of less toxic bile acids such as taurocholic acid.

Ischemic-type biliary lesions (ITBLs) post-orthotopic liver transplantation (OLT) are becoming an increasingly severe problem in the context of modern liver transplantation (Karimian et al. 2014). With a number of different surgical techniques available that reduce liver ischemia-reperfusion injury, a majority of graft loss problems develop due to biliary tree loss and subsequent cholestasis (Song et al. 2014). Anastomotic or non-anastomotic areas of biliary stricture result in a reduction in bile flow and severe cholestasis in post-OLT patients. Currently, there are only a minimal number of noninvasive diagnostic tests available for the diagnosis of biliary stricture, as endoscopic retrograde cholangiopancreatography (ERCP) is the primary diagnostic tool. Recent efforts have been put forth to identify potential prognostic assays. Biliary bile salt levels and biliary phospholipid excretion levels were both significantly different between patients with non-anastomotic strictures and patients without stricture over the first week post-OLT (Buis et al. 2009). Subsequent studies identified bile duct necrosis as a primary mechanism of biliary stricture, indicating that altered biliary phospholipid/ bile salt excretion levels might be indicative of biliary dysfunction and may serve as a future biomarker of cholangiopathies. Measurements of bile acids in bile both support the idea

that biliary bile acid levels may be exacerbating the ischemic toxicity and may yield some prognostic value (Buis et al. 2009). MicroRNA levels may also have some prognostic value as specific microRNAs are found in increased numbers in bile for patients with non-anastomotic stricture versus anastomotic stricture (Lankisch et al. 2014). Further investigation into the role of these miRNAs may yield some mechanistic insight into how ITBLs occur. Some evidence exists for a role for inflammation in biliary stricture development as well. IL-6 and interferon-gamma levels are both elevated in patients with biliary stricture versus control patients (Iacob et al. 2012). As primary sclerosing cholangitis as a cause of liver transplantation is a major risk factor for stricture development, the presence of pro-inflammatory chemokines and cytokines suggests inflammation may exacerbate injury and thus may be a useful therapeutic target.

These recent studies illustrate the use of mechanistic biomarkers in translational science. Future studies aimed at delineating mechanisms of inflammatory liver injury in cholestatic models using mechanistic biomarkers in human patients are warranted based on these findings. In addition, more work using serum biomarkers or more readily accessible tissue compartments may benefit the diagnosis of biliary stricture without invasive procedure.

Mechanistic Biomarkers in Drug-Induced Liver Injury

Drug-induced liver injury (DILI) is one of the most common causes of acute liver failure (ALF) in the world. DILI can be subdivided into idiosyncratic and intrinsic DILI. Idiosyncratic DILI is rare, unpredictable hepatotoxicity that occurs at therapeutic doses and generally has a delayed onset, while intrinsic DILI is highly predictable, dose-dependent toxicity (Jaeschke 2015) (Fig. 3). By far, the greatest single cause of DILI is overdose of acetaminophen (APAP), an intrinsic hepatotoxicant (Jaeschke 2015). Furthermore, the mechanisms of APAP-induced liver injury are relatively well understood. Thus, APAP toxicity is both a clinically important and experimentally useful model for the development and characterization of novel mechanistic biomarkers of liver injury. A number of mechanistic biomarkers have been measured in plasma or serum from APAP overdose patients (McGill and Jaeschke 2014). APAP-protein adducts are readily detectable in serum, even after therapeutic doses (Heard et al. 2011). It has been shown that several mitochondrial damage markers also increase after APAP overdose, namely, GLDH (McGill et al. 2012, 2014c), mtDNA (McGill et al. 2012, 2014c), and acylcarnitines (Bhattacharyya et al. 2014), although the latter may be affected by the standard-of-care treatment for APAP overdose, *N*-acetyl-cysteine (NAC) (McGill et al. 2014b; Bhattacharyya et al. 2014). CPS-1 has also been shown to increase in plasma from patients with APAP hepatotoxicity, and there is evidence that this protein is specifically released from mitochondria (Weerasinghe et al. 2014). Another biomarker for cell death identified in rodents and humans is argininosuccinate synthetase (ASS) (McGill et al. 2014a). Increases in ASS can be detected before ALT, but ASS levels decline more rapidly suggesting that ASS is a sensitive and more acute indicator of liver cell death (McGill et al. 2014a). Nuclear

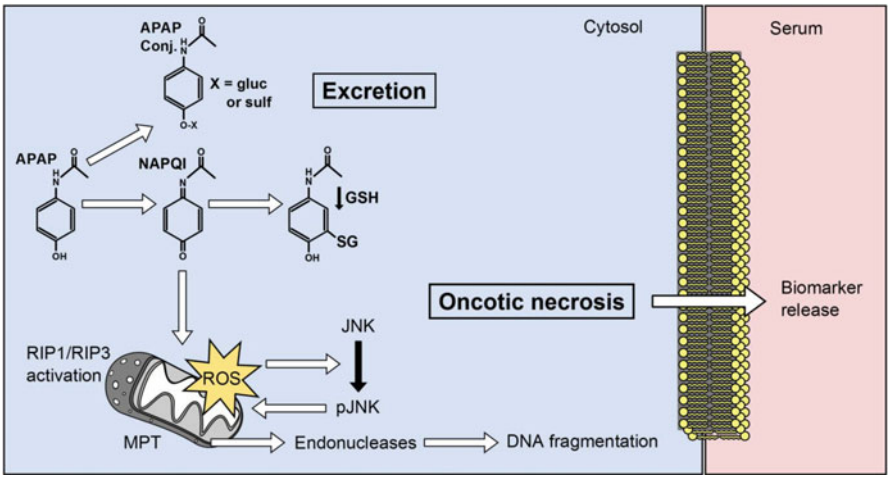

Fig. 3 Mechanisms of acetaminophen hepatotoxicity and biomarker release. Acetaminophen (APAP) is metabolized by two systems. Phase II conjugation enzymes are responsible for most APAP clearance. APAP is either glucuronidated or sulfated and can then be excreted in urine. Although phase I enzymes clear a smaller fraction, they also produce the reactive metabolite of APAP, *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI can be detoxified by glutathione (GSH), but also reacts with proteins. After an overdose, binding to mitochondrial proteins increases and this appears to play an important role in the toxicity. Reactive oxygen species (ROS) activate mitogen-activated protein kinase (MAPK) signaling pathways, including the c-Jun N-terminal kinase (JNK) pathway. Phosphorylated JNK 1/2 can then translocate into mitochondria and enhance ROS production. The receptor-interacting protein kinases 1 and 3 (RIP1 and RIP3) are induced and also appear to contribute to cell injury. Eventually, the mitochondrial permeability transition (MPT) pore opening occurs. Bax translocation and rupture of the outer mitochondrial membrane result in release of endonucleases from the intermembrane space. The endonucleases then fragment nuclear DNA. Ultimately, cell death occurs through oncotic necrosis, with release of various biomarkers (nuclear DNA fragments, mitochondrial DNA and proteins, alanine aminotransferase [ALT], etc.) into the extracellular fluid

DNA fragments are also increased in the circulation of APAP overdose patients (McGill et al. 2012). Interestingly, serum concentrations of GLDH, mtDNA, and nuclear DNA fragments all show a significant positive correlation with poor patient outcome in APAP-induced ALF (McGill et al. 2014c). The latter finding suggests that these biomarkers may be useful for patient prognosis and determination of the need for liver transplantation.

A number of other circulating mechanistic biomarkers have been measured in APAP overdose patients. Attempts to determine the mode of cell death revealed that caspase 3 cleavage and activity are undetectable in plasma after APAP overdose, despite the fact that both can be measured in the circulation of mice treated with TNF and galactosamine (McGill et al. 2012). Similarly, although some caspase-cleaved K18 can be detected in plasma from APAP overdose patients, it accounts for only a small percentage of the total K18 released (Antoine et al. 2012). The lack of caspase activation and the fact that most of the K18 in plasma after APAP overdose is intact

suggest that the primary mode of hepatic cell death during APAP toxicity is oncotic necrosis. Inflammation has also been assessed by measurement of cytokines (James et al. 2005), acetylated HMGB1 (Antoine et al. 2012), and both neutrophil (Williams et al. 2014) and monocyte (Antoniades et al. 2012) accumulation or activation. Together, the data seem to show that inflammation has an important role in APAP hepatotoxicity in humans, likely by enhancing liver regeneration (Jaeschke et al. 2012).

There has been limited use of mechanistic biomarkers to study other causes of DILI as well. For example, there is some evidence of oxidative stress in patients on antitubercular drugs (Walubo et al. 1995). The successful use of these biomarkers to better understand APAP hepatotoxicity in humans suggests that future translational studies of DILI could benefit from their application.

Mechanistic Biomarkers in Liver Ischemia-Reperfusion and Transplantation

Ischemia-reperfusion injury (IRI) is the process by which reintroduction of oxygen to a previously ischemic organ leads to exacerbation of injury to that organ (Fig. 4). IRI has been described in multiple organs including the liver, heart, and kidney. Ischemia can be classified as either "low flow" or "no flow" and further qualified as warm ischemia or cold ischemia. As the names suggest, "low-flow" ischemia involves sub-physiologic levels of blood flow to the liver, such as in conditions leading to severe hypotension or hypoxemia. On the other hand, no-flow ischemia involves a total cessation of blood flow to the liver, as is the case surgically during the Pringle maneuver or orthotopic liver transplantation (OLT). Interestingly, the liver tolerates prolonged periods of ischemia relatively well, but paradoxically, it is the return of blood flow to the tissue that exacerbates the injury. This is illustrated by the fact that liver injury, as measured by ALT, continues to increase long after the return of oxygen (Jaeschke and Woolbright 2012).

After decades of research, the mechanisms of IRI are fairly well described in the rodent (Fig. 4). During ischemia, relatively few cells undergo necrosis. During necrosis, the cell releases its contents, many of which act as damage-associated molecular patterns (DAMPs), into the sinusoids. These DAMPs subsequently activate the Kupffer cells (KCs) lining the sinusoids leading to secretion of inflammatory cytokines, primarily TNF-α (Jaeschke 2006; Lentsch 2012; Tsung et al. 2005). Increase in TNF-α leads to neutrophil recruitment, activation, and extravasation (Jaeschke 2006). Following extravasation, neutrophils, in an attempt to clear cellular debris, produce reactive oxygen species (ROS) which damage the hepatocyte (Jaeschke 2003). Under normal conditions, the levels of ROS produced by neutrophils are not high enough to cause cell death, but can cause the demise of an injured hepatocyte that may have otherwise survived (Jaeschke 2006). The hepatocytes killed by neutrophils release additional DAMPs, which leads to an injurious inflammatory cycle. In this way, the majority of liver injury is produced by the inflammatory response following reoxygenation (Jaeschke 2003; Jaeschke and Woolbright

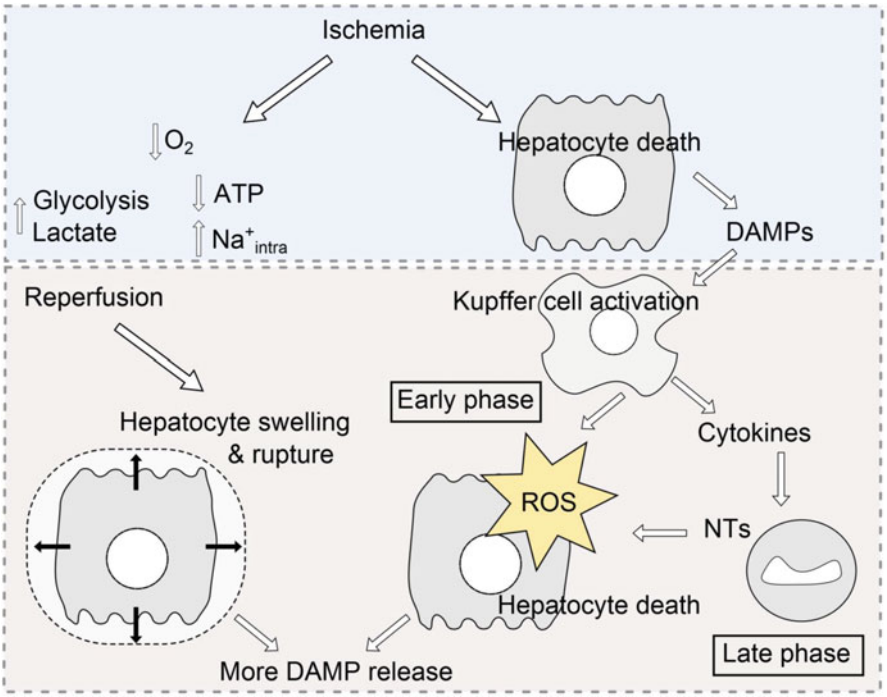

Fig. 4 Ischemia-reperfusion injury in the liver. Ischemia results in some hepatocyte death that results in release of damage-associated molecular patterns (DAMPs), which can activate immune cells. This can be exacerbated during the initial reperfusion period by the swelling and osmolysis of some hepatocytes. The latter is due to an increase in the intracellular concentrations of lactate and Na^+ that result from the switch to glycolysis that occurs during the low O_2 period. Upon reperfusion, fluid enters into the high-solute cells and causes membrane rupture. Upon activation, Kupffer cells can produce reactive oxygen species (ROS) that damage hepatocytes, as well as cytokines that recruit neutrophils (NTs) into the liver. This is the early phase of ischemia-reperfusion (I/R) injury. In the later phase, NTs enter the tissue and also produce ROS that damage hepatocytes

2012). In humans, however, much less is known regarding reperfusion injury. A major reason for this is that invasive biopsy samples cannot be acquired at multiple time points in patients undergoing liver surgery or transplantation. Therefore, it is not possible to view histology samples or perform simple laboratory experiments to identify the processes occurring. Thus, the use of biomarkers to describe these mechanisms has become an attractive approach.

Biomarkers in Warm Ischemia

In contrast to the research done on biomarkers in DILI, little has been done in the way of mechanistic biomarkers in IRI. In the rodent model of warm IRI, it appears as though biomarkers of both mode and mechanism of cell death can be used to describe

the molecular events occurring during IRI. For instance, following warm ischemia, there is a time-dependent increase in both ALT and miRNA-122, which peak around 24 h after reperfusion (Yang et al. 2014). Furthermore, the percent of acHMGB1 relative to the non-acetylated form increases dramatically at later time points (Gujral et al. 2001; Yang et al. 2014). This is in agreement with what multiple rodent studies have shown us – that the inflammatory response is mostly responsible for reperfusion injury (Jaeschke 2003). Furthermore, by evaluating levels of full-length and caspase-cleaved cytokeratin-18, it is evident that the primary mode of cell death during IRI is necrosis, rather than apoptosis (Yang et al. 2014). Determining the primary mode of cell death is of critical importance in designing treatment modalities to minimize injury following periods of ischemia. This pivotal study also demonstrated that the same biomarkers that can be used to describe DILI can be used to describe IRI in humans.

Hypoxic hepatitis (HH) is a clinical condition experienced fairly frequently, particularly in critically ill patients. During HH, there is decreased oxygen delivery to the liver, either due to decreased blood flow or hypoxemia or can be secondary to numerous causes, such as cardiogenic or hemorrhagic shock (Henrion et al. 2003). During HH, ALT values rapidly increase to greater than 20 times normal and gradually return to normal (Henrion et al. 2003; Horvatits et al. 2013). Although the mainstay of therapy in these patients is to treat the underlying cause, mortality still approaches approximately 50% (Horvatits et al. 2013). It is not a well-studied field, and a better understanding of the mechanisms involved could be helpful in identifying novel therapeutic approaches. Data from our laboratory suggest that the pattern of injury is very similar to that seen with acetaminophen toxicity (Weemhoff et al. 2016). In these patients, it appears cell death occurs via necrosis as evidenced by a greater than 25-fold elevation in full-length cytokeratin compared to caspase-cleaved cytokeratin. Interestingly, there is a steady increase in the percent of acHMGB1 relative to total HMGB1 following peak injury (Weemhoff et al. 2016). While this suggests a possible immune component, it is important to remember that in contrast to the rodent model of IRI in which only the liver is affected, in HH every organ can be affected, so any inflammation observed may not be specific to the liver. In vitro studies of hypoxemia and reoxygenation and in vivo models of hemorrhage and resuscitation suggest that mitochondria play a key role in mediating the injury though the formation of the MPTP and subsequent cell death (Lemasters et al. 1997). If this is the case during HH, levels of mitochondrial-specific biomarkers such as GDH and mtDNA would be expected to be present in serum. Interestingly, both of these biomarkers show a rapid increase followed by a steady decrease over several days (Weemhoff et al. 2016). These data provide further evidence to suggest that mitochondria are involved in injury, although whether these biomarkers are increased as a cause or simply as a consequence of HH needs to be studied further.

Biomarkers in Cold Ischemia

Liver transplantation is the only therapy for end-stage liver disease (ESLD) of any etiology. During transplantation, the donor organ is harvested, perfused with a

preservative (i.e., University of Wisconsin solution), and kept at hypothermic temperatures until it is placed into the recipient. In contrast to HH, transplantation is a relatively well-studied field. It is important to note here, however, that many studies extrapolate data from the rodent model of IRI to human liver transplantation. While both involve no-flow ischemia, the rodent model of IRI mostly used is a model of warm ischemia and warm reflow, which more accurately represent liver surgery in which the Pringle maneuver is employed. On the other hand, livers used in transplantation mainly undergo cold ischemia and warm reperfusion. As a result, the degree of injury is dramatically lower in these patients. While many studies have evaluated pre- and postoperative metabolic and inflammatory parameters in an attempt to identify markers predictive of outcome, only a few have attempted to measure the biomarkers discussed above. In these studies, the data suggests that during OLT, the primary mode of cell death is necrosis. This is supported by studies demonstrating a significant increase in miR-122 levels following reperfusion in transplanted livers (Farid et al. 2012). Furthermore, levels of tissue-derived levels of miR-122 decrease with an observed concomitant increase in serum levels, and this increase in serum levels mimics more conventional markers of hepatocellular necrosis such as ALT (Farid et al. 2012). Importantly, serum levels of miR-122 were shown to be elevated in patients experiencing acute rejection (Farid et al. 2012). Since miRNA has been shown to be a more sensitive indicator of hepatocellular necrosis than ALT, it may represent a promising biomarker for the early identification of patients who will experience AR. Early identification of these patients is of critical importance for the clinician. The idea that the primary mode of cell death occurs via necrosis following OLT is further supported by studies demonstrating that full-length cytokeratin-18 is significantly more elevated than caspase-cleaved cytokeratin at measured time points following OLT (Brenner et al. 2012). Increased cytokeratin levels have been associated with prolonged ischemic periods as well as subsequent increase in complications following OLT (Brenner et al. 2012), which suggests prolonged ischemic times are a factor in patient outcome. Despite this compelling data to the contrary, apoptosis is still thought by many to be the mode of cell death, and anti-apoptotic therapeutic strategies are being developed. The use of biomarkers to identify mechanisms of cell death is lacking. As in hypoxic hepatitis, it appears as though the mitochondria are targets of the injury as evidenced by increased GDH and mtDNA levels following reperfusion (Jaeschke and Weemhoff, unpublished data).

While the inflammatory response is known to play a crucial role in IRI in the rodent model of ischemia, its role in OLT is much less understood. While KC activation and neutrophil infiltration are known to exacerbate injury in the rodent model of IRI, there are conflicting reports as to their role in OLT (Ilmakunnas et al. 2009; Pesonen et al. 2000). Many studies have evaluated changing levels of various cytokines and chemokines following OLT, but these are difficult to interpret as many studies focus on similar cytokines but are measured at different time points. Additionally, as many cytokines have been shown to have both pro- and anti-inflammatory roles, descriptive

studies measuring only changes in cytokine levels at specific time points cannot accurately describe the contribution to liver injury. Further complicating interpretation of this data is the fact that even pro-inflammatory cytokines have been shown to be of benefit as they lead to the recruitment of phagocytes which clean cellular debris and promote tissue regeneration (Williams et al. 2014; Lentsch 2012). Further, whether these cytokines are being produced from activated KCs within the donor liver or from recipient-derived monocytes is unclear. Likely, it is a combination of both, but the degree to which each contributes to complications following OLT requires further examination.

In summary, the use of serum biomarkers represents an understudied but promising approach for the study of various models of IRI. As with other models of liver injury, these biomarkers achieve measurable levels in the serum and seem to recapitulate data from experimental models of IRI. However, further studies are needed before definitive conclusions can be made.

Potential Applications for Prognosis, Other Diseases, and Conditions

Several mechanistic biomarkers show promise for prognosis in various liver diseases. For example, miR-122, HMGB1, full-length K18, and ASS can be used to predict acute liver injury in APAP overdose patients presenting before an increase in serum ALT (McGill and Jaeschke 2014). Moreover, GLDH, mtDNA, nuclear DNA fragments, acetylated HMGB1, full-length K18, and the bile acid glycodeoxycholic acid are all increased in APAP-induced acute liver injury patients with poor outcome (Antoine et al. 2012; McGill et al. 2014c; Woolbright et al. 2014b). There is also evidence that cleaved K18 in serum reflects histology in nonalcoholic fatty liver disease (Vuppalanchi et al. 2014), and circulating levels of several microRNAs have been shown to correlate with survival in hepatocellular carcinoma (He et al. 2015). It is clear from these data that mechanistic biomarkers hold promise for clinical use.

Any of the biomarkers discussed in this chapter could be applied to the study of other diseases because they are not unique to the liver, but to the mechanisms of disease or injury. In fact, certain of these biomarkers were first measured in serum from patients with diseases in other tissues (e.g., Leers et al. 1999; Ueno et al. 2005; Zhang et al. 2010). There is direct evidence that mitochondrial damage biomarkers similar to mtDNA and GLDH can be applied to kidney disease. Whitaker et al. (2015) reported that they could measure the mitochondrial protein ATP synthase subunit-β in urine after acute kidney injury in both mice and humans and that the urinary levels of this biomarker correlated with evidence of reduced mitochondrial function in mice. It is likely that future research will continue to demonstrate the usefulness of mechanistic biomarkers for the study and monitoring of numerous diseases in both research and clinical practice.

Summary Points

- Existing clinical biomarkers of liver injury do not provide information about mechanisms and are generally not useful for prognosis.
- Mechanistic biomarkers provide information about the underlying mechanisms of tissue injury or disease.
- Mechanistic biomarkers may be useful for both diagnosis and prognosis in liver disease.
- Some mechanistic biomarkers are elevated in serum during acute liver injury, including DILI and ischemia-reperfusion injury.
- Some mechanistic biomarkers are elevated in chronic liver diseases, including alcoholic liver disease and cholestasis.
- Mechanistic biomarkers are becoming more widely used in research of diseases affecting other organs, including acute kidney injury and certain cancers.

References

Altara R, Manca M, Hermans KC, Daskalopoulos EP, Brunner-La Rocca HP, Hermans RJ, Struijker-Boudier HA, Blankesteijn MW. Diurnal rhythms of serum and plasma cytokine profiles in healthy elderly individuals assessed using membrane based multiplexed immunoassay. J Transl Med. 2015;13:129.

Antoine DJ, Jenkins RE, Dear JW, Wlliams DP, McGill MR, Sharpe M, Craig DG, Simpson KJ, Jaeschke H, Park BK. Molecular forms of HMGB1 and kertain-18 as mechanistic biomarkers for model of cell death and prognosis during clinical acetaminophen hepatotoxicity. J Hepatol. 2012;56(5):1070–9.

Antoniades CG, Quaglia A, Taams LS, Mitry RR, Hussain M, Abeles R, Possamai LA, Bruce M, McPhail M, Starling C, Wagner B, Barnardo A, Pomplun S, Auzinger G, Bernal W, Heaton N, Vergani D, Thursz MR, Wendon J. Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans. Hepatology. 2012;56(2):735–46.

Bajt ML, Lawson JA, Vonderfecht SL, Gujral JS, Jaeschke H. Protection against Fas receptor-mediated apoptosis in hepatocytes and nonparenchymal cells by a caspase-8 inhibitor in vivo: evidence for a postmitochondrial processing of caspase-8. Toxicol Sci. 2000;58(1):109–17.

Bajt ML, Cover C, Lemasters JJ, Jaeschke H. Nuclear translocation of endonuclease G and apoptosis-inducing factor during acetaminophen-induced liver cell injury. Toxicol Sci. 2006;94(1):217–25.

Bala S, Marcos M, Kodys K, Csak T, Catalano D, Mandrekar P, Szabo G. Up-regulation of microRNA-155 in macrophages contributes to increased tumor necrosis factor {alpha} (TNF {alpha}) production via increased mRNA half-life in alcoholic liver disease. J Biol Chem. 2011;286(2):1436–44.

Bala S, Petrasek J, Mundkur S, Catalano D, Levin I, Ward J, Alao H, Kodys K, Szabo G. Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. Hepatology. 2012;56(5):1946–57.

Beger RD, Bhattacharyya S, Yang X, Gill PS, Schnackenberg LK, Sun J, James LP. Translational biomarkers of acetaminophen-induced acute liver injury. Arch Toxicol. 2015;89(9):1497–522.

Bhattacharyya S, Yan K, Pence L, Simpson PM, Gill P, Letzig LG, Beger RD, Sullivan JE, Kearns GL, Reed MD, Marshall JD, Van Den Anker JN, James LP. Targeted liquid chromatography-

mass spectrometry analysis of serum acylcarnitines in acetaminophen toxicity in children. Biomark Med. 2014;8(2):147–59.
Boender PJ, Heijtink RA, Hellings JA. Nucleosomal fragments in serum may directly reflect cell-mediated cytotoxic activity in vivo. Clin Immonul Immunopathol. 1989;53(1):87–98.
Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, Rubartelli A, Agresti A, Bianchi ME. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards section. EMBO J. 2003;22(2):5551–60.
Brenner T, Rosenhagen C, Brandt H, Schmitt FCF, Jung GE, Schemmer P, Schmidt J, Mieth M, Bruckner T, Lichtenstern C, Martin EO, Weigand MA, Hofer S. Cell death biomarkers as early predictors for hepatic dysfunction in patients after orthotopic liver transplantation. Transplantation. 2012;94(2):185–91.
Buis CI, Geuken E, Visser DS, Kuipers F, Haagsma EB, Verkade HJ, Porte RJ. Altered bile composition after liver transplantation is associated with the development of nonanastomotic biliary strictures. J Hepatol. 2009;50(1):69–79.
Cazanave SC, Mott JL, Elmi NA, Bronk SF, Werneburg NW, Akazawa Y, Kahraman A, Garrison SP, Zambetti GP, Charlton MR, Gores GJ. NK1-dependent PUMA expression contributes to hepatocyte lipoapoptosis. J Biol Chem. 2009;284(39):26591–602.
Chen C, Krausz KW, Shah YM, Idle JR, Gonzalez FJ. Serum metabolomics reveals irreversible inhibition of fatty acid beta-oxidation through the suppression of PPARalpha activation as a contributing mechanism of acetaminophen-induced hepatotoxicity. Chem Res Toxicol. 2009; 22(4):699–707.
Cover C, Mansouri A, Knight TR, Bajt ML, Lemasters JJ, Pessayre D, Jaeschke H. Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. J Pharmacol Exp Ther. 2005;315(2):879–87.
Cusi K, Chang Z, Harrison S, Lomonaco R, Bril F, Orsak B, Ortiz-Lopez C, Hecht J, Feldstein AE, Webb A, Louden C, Goros M, Tio F. Limited value of plasma cytokeratin-18 as a biomarker for NASH and fibrosis in patients with non-alcoholic fatty liver disease. J Hepatol. 2014; 60(1):167–74.
De Vito R, Alisi A, Masotti A, Ceccarelli S, Panera N, Citti A, Salata M, Valenti L, Feldstein AE, Nobili V. Markers of activated inflammatory cells correlate with severity of liver damage in children with nonalcoholic fatty liver disease. Int J Mol Med. 2012;30(1):49–56.
Diab DL, Yerian L, Schauer P, Kashyap SR, Lopez R, Hazen SL, Feldstein AE. Cytokeratin 18 fragment levels as a noninvasive biomarker for nonalcoholic steatohepatitis in bariatric surgery patients. Clin Gastroenterol Hepatol. 2008;6(11):1249–54.
Duke RC, Chervenak R, Cohen JJ. Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytolysis. Proc Natl Acad Sci U S A. 1983;80(20):6361–5.
Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med. 2005;258(6):479–517.
Farid WR, Pan Q, van der Meer AJP, de Ruiter PE, Ramakrishnaiah V, de Jonge J, Kwekkeboom J, Janssen HL, Metselaar HJ, Tilanus HW, Kazemier G, van der Laan LJ. Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. Liver Transpl. 2012;18(3):290–7.
Farrell GC, van Rooyen D, Gan L, Chitturi S. NASH is an inflammatory disorder: pathogenic, prognostic and therapeutic implications. Gut Liver. 2012;6(2):149–71.
Feldstein AE, Canbay A, Angulo P, Taniai M, Burgart LJ, Lindor KD, Gores GJ. Hepatocyte apoptosis and Fas expression are prominent features of human nonalcoholic steatohepatitis. Gastroenterology. 2003;125(2):437–43.
Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. Gastroenterology. 2011;141(5):1572–85.
Ge X, Antoine DJ, Lu Y, Arriazu E, Leung TM, Klepper AL, Branch AD, Fiel MI, Nieto N. High mobility group box-1 (HMGB1) participates in the pathogenesis of alcoholic liver disease (ALD). J Biol Chem. 2014;289(33):22672–91.

Gujral JS, Bucci TJ, Farhood A, Jaeschke H. Mechanism of cell death during warm hepatic ischemia-reperfusion in rats: apoptosis or necrosis? Hepatology. 2001;33(2):397–405.
Gujral JS, Farhood A, Bajt ML, Jaeschke H. Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct-ligated mice. Hepatology. 2003;38(2):355–63.
Gujral JS, Liu J, Farhood A, Jaeschke H. Reduced oncotic necrosis in Fas receptor-deficient C57BL/6J-lpr mice after bile duct ligation. Hepatology. 2004;40(4):998–1007.
He S, Zhang C, Wei C. MicroRNAs as biomarkers for hepatocellular carcinoma diagnosis and prognosis. Clin Res Hepatol Gastroenterol. 2015;39(4):426–34.
Heard KJ, Green JL, James LP, Judge BS, Zolot L, Rhyees S, Dart RC. Acetaminophen-cysteine adducts during therapeutic dosing and following overdose. BMC Gastroenterol. 2011;11:20.
Henrion J, Schapira M, Luwaert R, Colin L, Delannoy A, Heller FR. Hypoxic hepatitis: clinical and hemodynamic study in 142 consecutive cases. Medicine. 2003;82(6):392–406.
Holdenrieder S, Stieber P, Chan LY, Geiger S, Kremer A, Nagel D, Lo YM. Cell-free DNA in serum and plasma: comparison of ELISA and quantitative PCR. Clin Chem. 2005;51(8):1544–6.
Horvatits T, Trauner M, Fuhrmann V. Hypoxic liver injury and cholestasis in critically ill patients. Curr Opin Crit Care. 2013;19(2):128–32.
Iacob S, Cicinnati VR, Dechêne A, Lindemann M, Heinemann FM, Rebmann V, Ferencik S, Sotiropoulos GC, Popescu I, Horn PA, Gerken G, Paul A, Beckebaum S. Genetic, immunological and clinical risk factors for biliary strictures following liver transplantation. Liver Int. 2012;32(8):1253–61.
Ilmakunnas M, Höckerstedt K, Mäkisalo H, Siitonen S, Repo H, Pesonen EJ. Hepatic neutrophil activation during reperfusion may not contribute to initial graft function after short cold ischemia in human liver transplantation. Transplant Proc. 2009;41(2):739–42.
Iracheta-Vellve A, Petrasek J, Satishchandran A, Gyongyosi B, Saha B, Kodys K, Fitzgerald KA, Kurt-Jones EA, Szabo G. Inhibition of sterile danger signals, uric acid and ATP, prevents inflammasome activation and protects from alcoholic steatohepatitis in mice. J Hepatol. 2015;63(5):1147–55.
Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. Am J Physiol Gastrointest Liver Physiol. 2003;284(1):G15–26.
Jaeschke H. Mechanisms of liver injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. Am J Physiol Gastrointest Liver Physiol. 2006;290(6):G1083–8.
Jaeschke H. Acetaminophen: dose-dependent drug hepatotoxicity and acute liver failure in patients. Dig Dis. 2015;33(4):464–71.
Jaeschke H, Woolbright BL. Current strategies to minimize hepatic ischemia-reperfusion injury by targeting reactive oxygen species. Transplant Rev (Orlando). 2012;26(2):103–14.
Jaeschke H, Williams CD, Ramachandran A, Bajt ML. Acetaminophen hepatotoxicity and repair: the role of sterile inflammation and innate immunity. Liver Int. 2012;32(1):8–20.
James LP, Simpson PM, Farrar HC, Kearns GL, Wasserman GS, Blumer JL, Reed MD, Sullivan JE, Hinson JA. Cytokines and toxicity in acetaminophen overdose. J Clin Pharmacol. 2005; 45(10):1165–71.
Karimian N, Westerkamp AC, Porte RJ. Biliary complications after orthotopic liver transplantation. Curr Opin Organ Transplant. 2014;19(3):209–16.
Lankisch TO, Voigtländer T, Manns MP, Holzmann A, Dangwal S, Thum T. MicroRNAs in the bile of patients with biliary strictures after liver transplantation. Liver Transpl. 2014; 20(6):673–8.
Lavallard VJ, Bonnafous S, Patouraux S, Saint-Paul MC, Rousseau D, Anty R, Le Marchand-Brustel Y, Tran A, Gual P. Serum markers of hepatocyte death and apoptosis are non-invasive biomarkers of severe fibrosis in patients with alcoholic liver disease. PLoS One. 2011;6(3), e17599.
Leers MP, Kölgen W, Björklund V, Bergman T, Tribbick G, Persson B, Björklund P, Ramaekers FC, Björklund B, Nap M, Jörnvall H, Schutte B. Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. J Pathol. 1999;187(5):567–72.

Lemasters JJ, Nieminen AL, Qian T, Trost LC, Herman B. The mitochondrial permeability transition in toxic, hypoxic and reperfusion injury. Mol Cell Biochem. 1997;174(1–2): 159–65.

Lentsch AB. Regulatory mechanisms of injury and repair after hepatic ischemia/reperfusion. Scientifica (Cairo). 2012;2012:513192.

Lippai D, Bala S, Catalano D, Kodys K, Szabo G. Micro-RNA-155 deficiency prevents alcohol-induced serum endotoxin increase and small bowel inflammation in mice. Alcohol Clin Exp Res. 2014;38(8):2217–24.

Malhi H, Guicciardi ME, Gores GJ. Hepatocyte death: a clear and present danger. Physiol Rev. 2010;90(3):1165–1194.

Marcellin P, Martinot M, Boyer N, Lévy S. Treatment of hepatitis C patients with normal aminotransferase levels. Clin Liver Dis. 1999;3(4):843–53.

McDaniel K, Herrera L, Zhou T, Francis H, Han Y, Levine P, Lin E, Glaser S, Alpini G, Meng F. The functional role of microRNAs in alcoholic liver injury. J Cell Mol Med. 2014; 18(2):197–207.

McGill MR, Jaeschke H. Mechanistic biomarkers in acetaminophen-induced hepatotoxicity and acute liver failure: from preclinical models to patients. Expert Opin Drug Metab Toxicol. 2014;10(7):1005–17.

McGill MR, Jaeschke H. MicroRNAs as signaling mediators and biomarkers of drug- and chemical-induced liver injury. J Clin Med. 2015;4(5):1063–78.

McGill MR, Sharpe MR, Williams CD, Taha M, Curry SC, Jaeschke H. The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. J Clin Invest. 2012;122(4):1574–83.

McGill MR, Cao M, Svetlov A, Sharpe MR, Williams CD, Curry SC, Farhood A, Jaeschke H, Svetlov SI. Argininosuccinate synthetase as a plasma biomarker of liver injury after acetaminophen overdose in rodents and humans. Biomarkers. 2014a;19(3):222–30.

McGill MR, Li F, Sharpe MR, Williams CD, Curry SC, Ma X, Jaeschke H. Circulating acylcarnitines as biomarkers of mitochondrial dysfunction after acetaminophen overdose in mice and humans. Arch Toxicol. 2014b;88(2):391–401.

McGill MR, Staggs VS, Sharpe MR, Lee WM, Jaeschke H, Acute Liver Failure Study Group. Serum mitochondrial biomarkers and damage-associated molecular patterns are higher in acetaminophen overdose patients with poor outcome. Hepatology. 2014c;60(4):1336–45.

Miller TJ, Knapton A, Adeyemo O, Noory L, Weaver J, Hanig JP. Cytochrome c: a non-invasive biomarker of drug-induced liver injury. J Appl Toxicol. 2008;28(7):815–28.

Moldawer LL, Gelin J, Scherstén T, Lundholm KG. Circulating interleukin 1 and tumor necrosis factor during inflammation. Am J Pathol. 1987;253(6 Pt 2):R922–8.

Momen-Heravi F, Bala S, Kodys K, Szabo G. Exosomes derived from alcohol-treated hepatocytes horizontally transfer liver specific miRNA-122 and sensitize monocytes to LPS. Sci Rep. 2015;5:9991.

O'Donnell MA, Legarda-Addison D, Skountzos P, Yeh WC, Ting AT. Ubiquitination of RIP1 regulates an NF-kappaB-independent cell-death switch in TNF signaling. Curr Biol. 2007; 17(5):418–24.

O'Grady JG, Alexander GJ, Hayllar KM, Williams R. Early indicators of prognosis in fulminant hepatic failure. Gastroenterology. 1989;97(9):439–45.

Panteghini M, Malchiodi A, Calarco M, Bonora R. Clinical and diagnostic significance of aspartate aminotransferase isoenzymes in sera of patients with liver diseases. J Clin Chem Clin Biochem. 1984;22(2):153–8.

Pesonen EJ, Höckerstedt K, Mäkisalo H, Vuorte J, Jansson SE, Orpana A, Karonen SL, Repo H. Transhepatic neutrophil and monocyte activation during clinical liver transplantation. Transplantation. 2000;69(7):1458–64.

Rigamonti C, Mottaran E, Reale E, Rolla R, Cipriani V, Capelli F, Boldorini R, Vidali M, Sartori M, Albano E. Moderate alcohol consumption increases oxidative stress in patients with chronic hepatitis C. Hepatology. 2003;38(1):42–9.

Royo F, Falcon-Perez JM. Liver extracellular vesicles in health and disease. J Extracell Vesicles. 2012;1:1–7.
Schüngel S, Buitrago-Molina LE, Nalapareddy PD, Lebofsky M, Manns MP, Jaeschke H, Gross A, Vogel A. The strength of the Fas ligand signal determines whether hepatocytes act as type 1 or type 2 cells in murine livers. Hepatology. 2009;50(5):1558–66.
Shigenaga MK, Gimeno CJ, Ames BN. Urinary 8-hydroxy-2′-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. Proc Natl Acad Sci U S A. 1989;86(24):9697–701.
Singh R, Wang Y, Xiang Y, Tanaka KE, Gaarde WA, Czaja MJ. Differential effects of JNK1 and JNK2 inhibition on murine steatohepatitis and insulin resistance. Hepatology. 2009; 49(1):87–96.
Song GW, Lee SG, Hwang S, Kim KH, Ahn CS, Moon DB, Ha TY, Jung DH, Park GC, Kang SH, Jung BH, Yoon YI, Kim N. Biliary stricture is the only concern in ABO-incompatible adult living donor liver transplantation in the rituximab era. J Hepatol. 2014;61(3):575–82.
Tamimi TI, Elgouhari HM, Alkhouri N, Yerian LM, Berk MP, Lopez R, Schauer PR, Zein NN, Feldstein AE. An apoptosis panel for nonalcoholic steatohepatitis diagnosis. J Hepatol. 2011; 54(6):1224–9.
Thapaliya S, Wree A, Povero D, Inzaugarat ME, Berk M, Dixon L, Papouchado BG, Feldstein AE. Caspase 3 inactivation protects against hepatic cell death and ameliorates fibrogenesis in a diet-induced NASH model. Dig Dis Sci. 2014;59(6):1197–206.
Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, Yang H, Li J, Tracey KJ, Geller DA, Billiar TR. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med. 2005;201(7):1135–43.
Ueno T, Toi M, Linder S. Detection of epithelial cell death in the body by cytokeratin 18 measurement. Biomed Pharmacother. 2005;59 Suppl 2:S359–62.
Van Hove JL, Zhang W, Kahler SG, Roe CR, Chen YT, Terada N, Chace DH, Iafolla AK, Ding JH, Millington DS. Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency: diagnosis by acylcarnitine analysis in blood. Am J Hum Genet. 1993;52(5):958–66.
Vuppalanchi R, Jain AK, Deppe R, Yates K, Comerford M, Masouka HC, Neuschwander-Tetri BA, Loomba R, Brunt EM, Kleiner DE, Molleston JP, Schwimmer JB, Lavine JE, Tonascia J, Chalasani N. Relationship between changes in serum levels of keratin 18 and changes in liver histology in children and adults with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol. 2014;12(12):2121–30.
Walenbergh SM, Houben T, Hendrikx T, Jeurissen ML, van Gorp PJ, Vreugdenhil AC, Adriaanse MP, Buurman WA, Hofker MH, Mosca A, Lindsey PJ, Alisi A, Liccardo D, Panera N, Koek GH, Nobili V, Shiri-Sverdlov R. Plasma cathepsin D levels: a novel tool to predict pediatric hepatic inflammation. Am J Gastroenterol. 2015;110(3):462–70.
Walubo A, Smith PJ, Folb PI. Oxidative stress during antituberculosis therapy in young and elderly patients. Biomed Environ Sci. 1995;8(2):106–13.
Ward J, Kanchagar C, Veksler-Lublinsky I, Lee RC, McGill MR, Jaeschke H, Curry SC, Ambros VR. Circulating microRNA profiles in human patients with acetaminophen hepatotoxicity or ischemic hepatitis. Proc Natl Acad Sci U S A. 2014;111(33):12169–74.
Weemhoff JL, Woolbright BL, Jenkins RE, McGill MR, Sharpe MR, Olson JC, Antoine DJ, Curry SC, Jaeschke H. Plasma biomarkers to study mechanisms of liver injury in patients with hypoxic hepatitis. Liver Int. 2016. doi:10.1111/liv.13202 [Epub ahead of print].
Weerasinghe SV, Jang YJ, Fontana RJ, Omary MB. Carbamoyl phosphate synthetase-1 is a rapid turnover biomarker in mouse and human acute liver injury. Am J Physiol Gastrointest Liver Physiol. 2014;307(3):G355–64.
Whitaker RM, Korrapati MC, Stallons LJ, Jesinkey SR, Arthur JM, Beeson CC, Zhong Z, Schnellmann RG. Urinary ATP synthase subunit β is a novel biomarker of renal mitochondrial dysfunction in acute kidney injury. Toxicol Sci. 2015;145(1):108–17.
Williams CD, Bajt ML, Sharpe MR, McGill MR, Farhood A, Jaeschke H. Neutrophil activation during acetaminophen hepatotoxicity and repair in mice and humans. Toxicol Appl Pharmacol. 2014;275(2):122–33.

Woolbright BL, Jaeschke H. Novel insight into mechanisms of cholestatic liver injury. World J Gastroenterol. 2012;18(36):4985–93.

Woolbright BL, Antoine DJ, Jenkins RE, Bajt ML, Park BK, Jaeschke H. Plasma biomarkers of liver injury and inflammation demonstrate a lack of apoptosis during obstructive cholestasis in mice. Toxicol Appl Pharmacol. 2013;273(3):524–31.

Woolbright BL, Li F, Xie Y, Farhood A, Fickert P, Trauner M, Jaeschke H. Lithocholic acid feeding results in direct hepato-toxicity independent of neutrophil function in mice. Toxicol Lett. 2014a;228(1):56–66.

Woolbright BL, McGill MR, Staggs VS, Winefield RD, Gholami P, Olyaee M, Sharpe MR, Curry SC, Lee WM, Jaeschke H, Acute Liver Failure Study Group. Glycodeoxycholic acid levels as prognostic biomarker in acetaminophen-induced acute liver failure patients. Toxicol Sci. 2014b;142(2):436–44.

Woolbright BL, Dorko K, Antoine DJ, Clarke JI, Gholami P, Li F, Kumer SC, Schmitt TM, Forster J, Fan F, Jenkins RE, Park BK, Hagenbuch B, Olyaee M, Jaeschke H. Bile acid-induced necrosis in primary human hepatocytes and in patients with obstructive cholestasis. Toxicol Appl Pharmacol. 2015;283(3):168–77.

Xie Y, McGill MR, Du K, Dorko K, Kumer SC, Schmitt TM, Ding WX, Jaeschke H. Mitochondrial protein adducts formation and mitochondrial dysfunction during N-acetyl-m-aminophenol (AMAP)-induced hepatotoxicity in primary human hepatocytes. Toxicol Appl Pharmacol. 2015;289(2):213–22.

Yang M, Antoine DJ, Weemhoff JL, Jenkins RE, Farhood A, Park BK, Jaeschke H. Biomarkers distinguish apoptotic and necrotic cell death during hepatic ischemia-reperfusion injury in mice. Liver Transplant. 2014;20(11):1372–82.

Yang X, Salminen WF, Shi Q, Greenhaw J, Gill PS, Bhattacharyya S, Beger RD, Mendrick DL, Mattes WB, James LP. Potential of extracellular microRNAs as biomarkers of acetaminophen toxicity in children. Toxicol Appl Pharmacol. 2015;284(2):180–7.

Yilmaz Y, Dolar E, Ulukaya E, Akgoz S, Keskin M, Kiyici M, Aker S, Yilmaztepe A, Gurel S, Gulten M, Nak SG. Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from nonalcoholic steatohepatitis. World J Gastroenterol. 2007;13(6):837–44.

Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, Hauser CJ. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature. 2010;464(7285):104–7.

Zhang Y, Hong JY, Rockwell CE, Copple BL, Jaeschke H, Klaassen CD. Effect of bile duct ligation on bile acid composition in mouse serum and liver. Liver Int. 2012;32(1):58–69.

Zhang X, Shen J, Man K, Chu ES, Yau TO, Sung JC, Go MY, Deng J, Lu L, Wong VW, Sung JJ, Farrell G, Yu J. CXCL10 plays a key role as an inflammatory mediator and a non-invasive biomarker of non-alcoholic steatohepatitis. J Hepatol. 2014;61(6):1365–75.

Liver Transplantation Biomarkers in the Metabolomics Era

5

Miriam Cortes, Juan Carlos García-Cañaveras, Eugenia Pareja, and Agustín Lahoz

Contents

Abstract

The term biomarker usually refers to the biochemical molecules used in basic and clinical research, and also in the clinical practice, as surrogate markers that offer the advantage of being an objective, quantifiable, and reproducible measure. The

M. Cortes
Liver Transplant Unit, Institute of Liver Studies, King's College Hospital, London, UK
e-mail: corcer_miriam@yahoo.es

J.C. García-Cañaveras • A. Lahoz (✉)
Unidad de Hepatología Experimental, Unidad Analítica, Instituto de Investigación Sanitaria, Fundación Hospital La Fe, Valencia, Spain
e-mail: j.carlos.garcia@uv.es; agustin.lahoz@uv.es

E. Pareja
Unidad de Hepatología Experimental, Unidad Analítica, Instituto de Investigación Sanitaria, Fundación Hospital La Fe, Valencia, Spain

Unidad de Cirugía Hepato-Bilio-Pancreatica y Trasplante Hepático, Hospital Universitario y Politécnico La Fe de Valencia, Valencia, Spain
e-mail: pareja_eug@gva.es

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_42

most common applications of biomarkers include diagnosis, screening and monitoring of disease, assessment of response during therapy, risk assessment, and prognosis. Metabolomics or metabonomics enables the determination of hundreds of small molecules at the same time, which provides more comprehensive information than the determination of a single biomarker. Using metabolomics as an approach for searching biomarkers is supported by its capabilities to detect subtle metabolic changes triggered by external stimuli or perturbation. Metabolome changes are quite dynamic compared to genomics and transcriptomics, or even proteomics. Therefore, such metabolite alterations are found early in different samples, like tissues, cell lysates, blood, serum, plasma, feces, urine, etc. Application of metabolomics in liver transplantation is still in its early stages and has focused mainly on studying three aspects: post-reperfusion damage and rejection and dysfunction of the organ. In the current era when lack of organs suitable for transplantation is the most important limiting factor, the existence of an accepted functional assessment of grafts before transplantation would help to not only recover initially discarded organs but to also assess the therapies used to improve the quality of these organs. Different metabolic approaches have been used to search for objective markers of graft function and quality, but further analytical and clinical validation in multicentre studies is mandatory before they are incorporated into clinical routines.

Keywords

Omics • Biomarkers • Metabonomics • Metabolomics • Mass spectrometry • Bile acids • Phospholipids • Liver transplant • Ischemia reperfusion injury • Graft dysfunction

List of Abbreviations

AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ADMA	Asymmetric dimethylarginine
BA	Bile acids
DBD	Donation after brain dead
DCD	Donation after circulatory dead
C18	Aliphatic chain of length 18
CEAD	Colorimetric electrochemical array detection
CIT	Cold ischemia time
CS	Cold storage
EAD	Early allograft dysfunction
ECD	Extended criteria donor
FT-ICR MS	Fourier transform ion cyclotron resonance mass spectrometry
FXR	Farnesoid X receptor
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography

HR-MAS H-NMR	High-resolution magic angle spinning
H-NMR	Proton nuclear magnetic resonance spectroscopy
IGF	Initial good function
IP-LC	Ion-pairing liquid chromatography
IRI	Ischemia reperfusion injury
JNK	c-Jun N-terminal kinase
LC	Liquid chromatography
LT	Liver transplant
MRM	Multiple reaction monitoring
NIH	National Institute of Health
NO	Nitric oxide
NOS	Nitric oxide synthase
NMP	Normothermic machine perfusion
OPLS-DA	Orthogonal projection to latent structures-discriminant analysis
PCA	Principal component analysis
PLS	Partial least square
PLS-DA	Partial least square-discriminant analysis
PNF	Primary non-function
QC	Quality control
Q-ToF	Quadrupole time of flight
ROS	Reactive oxygen species
Rt	Retention time
RP	Reversed phase
TQ	Triple quadrupole
UPLC	Ultraperformance liquid chromatography
WHO	World Health Organization

Key Facts of Metabolomics

- Metabolomics/metabonomics can be defined as the holistic determination of low-molecular-weight (<1.5 kDa) molecules present in a biological system (cell, tissue, or organism).
- According to the Human Metabolome Database, human metabolome size is estimated to be composed of around 42,000 endogenous and exogenous metabolites, including lipids, small peptides, carbohydrates, cofactors, amino acids, etc.
- As a result of being downstream of the activity of genes and proteins, the metabolome constitutes a closer approach to the phenotype than genes and transcripts, or even proteins.
- The most common analytical techniques used to study the metabolome are nuclear magnetic resonance spectroscopy and mass spectrometry. The latter is usually coupled to previous separation techniques, such as liquid chromatography, gas chromatography, and capillary electrophoresis.
- MS-based metabolomics can be performed by two different approaches: (i) untargeted metabolomics, which aims to determine the global metabolomic

profile, and (ii) targeted metabolomics, where only a subset of the metabolome is determined.
- From a human perspective, metabolomics can be applied to body tissues or fluids, and selection strongly conditions the metabolites that are expected to be detected and the meaning of the altered metabolomic patterns.
- Metabolomic studies in liver organ transplants have focused on three aspects: (i) ischemia reperfusion injury, (ii) graft dysfunction, and (iii) assessment of donor liver quality before transplantation.

Definitions of Words and Terms

Biomarker	The characteristic that is objectively evaluated as an indicator of normal biological and pathogenic processes or pharmacological responses to a therapeutic intervention.
Cold ischemia time	The period from the time the organ is perfused in the donor with preservation solution until organ implantation starts in the recipient.
Donor after circulatory death	Organ donors in which the death is certified after cardiac arrest.
Donors after brain death	Organ donors after the diagnosis of brain stem death.
Early allograft dysfunction	It is the dysfunction of the organ after being transplanted not related to other causes such as vascular complications, infection, or rejection.
Extended criteria donors	Organ donors with characteristics beyond standard limits that may compromise the outcome in the recipient after using his/her organs.
Ischemia reperfusion injury	The damage that occurs within the transplanted graft when it is reperfused in the recipient after an ischemia period in cold storage.
Metabolite	Low-molecular-weight molecules ($<$1.5 kDa) that are the intermediates of biochemical reactions.
Metabolome	Collection of all the metabolites present in a given biological system (i.e., cell, biofluid, tissue, organism, etc.).
Metabolomics	The comprehensive and quantitative analysis of all the metabolites present in a specific cellular, tissue, or biological sample.
Metabonomics	The quantitative measurement of the dynamic multiparametric metabolic response of living

	systems to pathophysiological stimuli or genetic modification.
Targeted metabolomics	Guided (quantitative) determination of a predefined set of metabolites of interest.
Untargeted metabolomics	Holistic/global determination of the metabolites present in a given biological specimen.
Warm ischemia time	The period from when organ implantation starts until it is reperfused in the recipient.

Introduction

The term "biomarker" results from the combination of the terms "biological marker" and has been defined by the National Institute of Health (NIH) as "the characteristic that is objectively evaluated as an indicator of normal biological and pathogenic processes or pharmacological responses to a therapeutic intervention" (Biomarkers Definition Working Group 2001). A broader definition by the World Health Organization (WHO) has defined biomarker as "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction" (WHO International Programme on Chemical Safety 1993). The utilization of small molecule measurement has been a common clinical practice in the field of medicine for more than 100 years (Wishart 2005); biochemical measurements of urine glucose, serum creatinine, or urea are still a very useful tool in the clinical practice. Nowadays, the term biomarker mostly refers to molecular or biochemical molecules which have become surrogate markers in basic and clinical research and also in clinical practice and have the advantage of being an objective, quantifiable, and reproducible measure (Strimbu and Tavel 2010). Biomarkers offer different applications in the field of human health, not only once the disease is present but also to predict the future. The intended uses of biomarkers comprise: (i) diagnosis of symptomatic patients; (ii) detection or screening of disease by enabling intervention in an earlier and potentially more curable stage than under usual clinical diagnostic conditions; (iii) monitoring disease, following the response during therapy, with the potential for adjusting the level of intervention (e.g., dose) on a dynamic and personal basis; (iv) risk assessment, which leads to preventive interventions for those at sufficient risk; (v) prognosis, which allows to adjust therapy (more or less aggressive) for patients according to the expected prognosis; (vi) prediction, which provides guidance in selecting specific therapy for patients or tailoring its dose for safety and efficacy purposes.

The term "omics" refers to a field of science defined as "the molecular or biochemical characterisation of pools of biological molecules, such as genes and genomes, transcripts and transcriptomes, proteins and proteomes, and small molecules, metabolites and metabolomes, which together encode the structure and

function of an organism or organisms, and can be used to explore their dynamics and flexibilities" (Gilbert et al. 2014). "Omics" technologies (i.e., genomics, transcriptomics, proteomics, and metabolomics) are characterized by the simultaneous determination of multiple parameters in a single biological sample (Gomez-Lechon et al. 2010). Metabolomics measures the downstream products of the "omics cascade" and thus provides information that is not accessible through other alternative "omics," such as genomics, transcriptomics, or proteomics (Dettmer et al. 2007; Leon et al. 2013). Metabolites are the intermediates of biochemical reactions. Hence their levels are defined by both their concentration and the functional properties of the enzymes, where the latter are the result of the integration of transcription, translation, posttranslational modifications, and allosteric effects, which results in an integrative effect between the capabilities of the system under study and its interaction with the environment (Villas-Boas et al. 2005). As a result of being downstream of the activity of genes and proteins, the metabolome constitutes a closer approach to the phenotype than genes, transcripts, or proteins. So metabolomics is more informative of the functional status of cells than other "omics" agents (Kaddurah-Daouk et al. 2008; Fig. 1). While common biochemical analyses also assess levels of metabolites, the main difference compared to metabolomics is that the latter measures hundreds of small molecules at the same

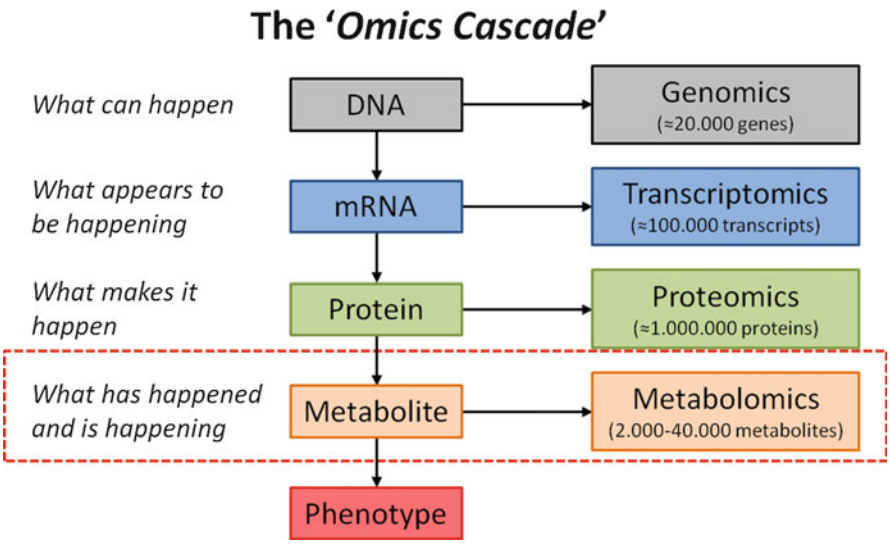

Fig. 1 The "omics cascade" in systems biology. Omics sciences provide a complete overview of the system under study. Biological information goes from genes (genomics) that provide information about what can happen through mRNA (transcriptomics) and proteins (proteomics), which provide information about what appears to happen and what makes it happen, respectively, and finally to metabolites (metabolomics), which represent the perturbations of the genome, transcriptome, and proteome and, therefore, provide information of what has happened and what is happening

time, which provides much more information than the simple determination of a single marker (Wishart 2005).

Metabolomics

The main idea behind metabolomics is that diseases produce changes in body fluids and tissues, which was already known in ancient Greece, and is represented in the diagnostic "urine charts" used since the Middle Ages, where the colors, smells, and tastes of urine were related to various medical conditions. Indeed gas chromatography (GC) and ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR) have been employed to perform the metabolic profiling of biological samples for decades (Nicholson and Lindon 2008). Despite the formal definitions of metabolome, which refer to the quantitative complement of all the low-molecular-weight molecules present in a cell in a particular physiological or developmental state (Oliver et al. 1998), metabonomics, defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al. 1999), or metabolomics, defined as "the comprehensive and quantitative analysis of all the metabolites present in a specific cellular, tissue, or biological sample," are much more recent. In generic terms, metabonomics/metabolomics can be defined as the holistic determination of the low-molecular-weight (<1.5 kDa) molecules present in a biological system (cell, tissue, or organism), such as lipids, small peptides, carbohydrates, cofactors, amino acids, etc. Human metabolome size is estimated as being composed of around 42,000 endogenous and exogenous metabolites according to the Human Metabolome Database (http://www.hmdb.ca/) (Wishart et al. 2013). The number of major metabolites, that is, those at higher concentrations and with the most relevant functions, is estimated to be around 2,000 (Beecher 2003). In order to provide a couple of examples, the human urine metabolome is estimated to be composed of 3,100 compounds, with the predominance of highly polar compounds (Bouatra et al. 2013), while the human serum metabolome is estimated to be composed of around 4,600 metabolites, half of which are phospholipids, and over a 1,000 glycerolipids (Psychogios et al. 2011).

Metabolomics Workflow

The basic working approach with any "-omic" science, including metabolomics, is shown in Fig. 2. Given their relevance, certain aspects of this approach must be highlighted. The experimental design is key in any metabolomic study because of the vast experimental variability that an inadequate design can imply. Some noteworthy aspects are defining the cohort study, selecting the type of biological samples of interest to perform the analysis, deciding sample treatment/processing, choosing the analytical method, etc.

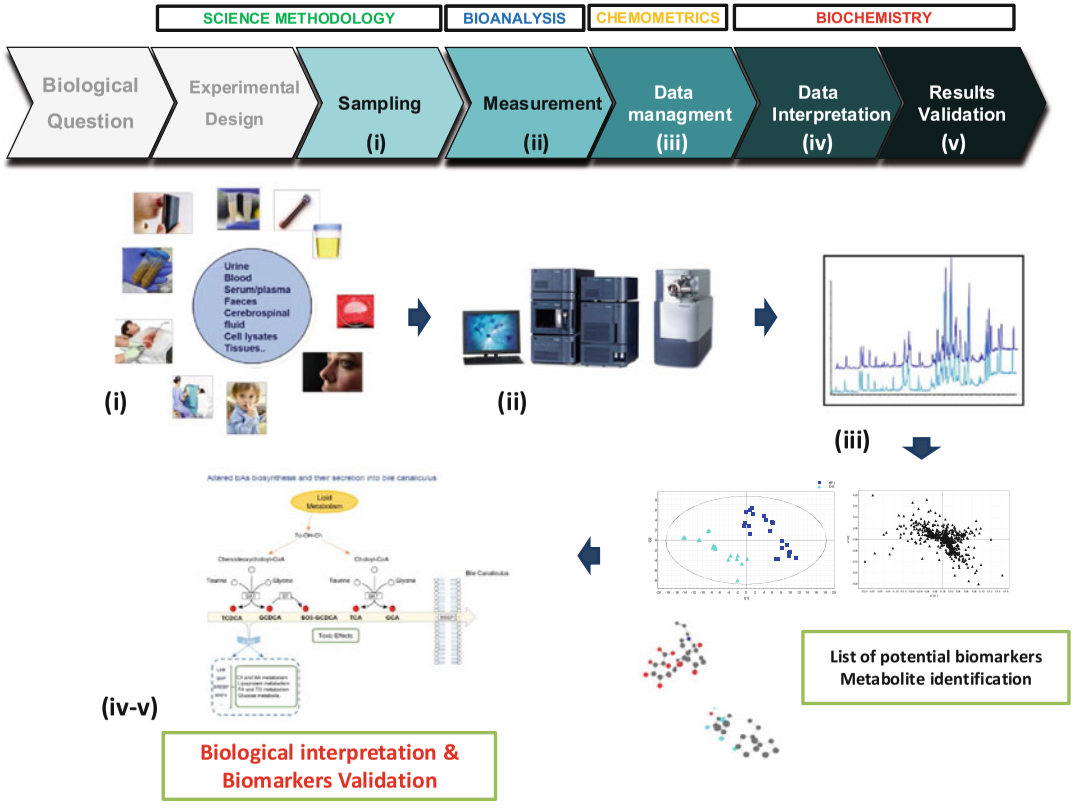

Fig. 2 A general metabolomic workflow. The figure represents the different steps involved in the sample metabolomics analysis, from sample collection and preparation *i*) to validating the results (*v*) through metabolite determination (*ii*) and data analyses (*iii-iv*)

Samples Most Widely Used in Metabolomics

Metabolomics, when referring to human samples, can be applied to body tissues or fluids. While the metabolic profiling of tissues is expected to more closely reflect their function, most of the current data available in the literature correspond to metabolomic analyses in fluids, probably because they can be obtained without resorting to invasive procedures (Dunn et al. 2005). For biofluids, metabolite levels not only reflect the status of the organ of biosynthesis but are dependent on other several factors at an organism's level. Sample preparation is a key step in a metabolomic study as it strongly conditions the results, and several factors have to be evaluated at both the time of sampling and during sample processing as they can affect their quality, such as type of sample, the containers used to store them, storage time, the necessity to add any preservatives, and the time taken to process them (Holland et al. 2003). While the most common procedure for body fluids is to perform protein precipitation using an organic solvent, with tissues, metabolites have to be efficiently extracted. In any case, sample processing should avoid potential interferences, ensure minimal loss of metabolites, and be compatible with subsequent analytical procedures (Leon et al. 2013).

Platforms Used to Measure the Metabolome

The metabolome presents a high diversity of components (amino acids, carbohydrates, lipids, organic acids, etc.) with very different chemical structures and properties (from ionic or very polar to highly hydrophobic compounds). It is almost impossible to determine the complete metabolome using a single analytical platform, thus the combination of complementary techniques (covering both sample preparation and analysis) is required to achieve comprehensive metabolome coverage (Villas-Boas et al. 2005; Dettmer et al. 2007). The most frequent analytical techniques used to study the metabolome are NMR spectroscopy and mass spectrometry (MS), the latter is usually hyphenated to previous separation techniques (Robertson 2005). NMR and MS, in their different configurations, are complementary rather than opposite platforms (Table 1). Thus the use of different analytical techniques has a positive impact on widening metabolome coverage (Leon et al. 2013).

^{1}H-NMR spectroscopy is based on the detection of all the proton signals present in a given sample. The main advantages of NMR spectroscopy are: (i) it is a nondestructive technique; therefore, samples may be used in further analyses; (ii) it requires no or little sample preparation; (iii) it is possible to perform analyses with solid samples; (iv) it is an intrinsically quantitative technique; (v) it is possible to perform structural analyses; (vi) its high robustness allows easy lab-to-lab comparisons. However, the NMR application is hampered by its low resolution and sensitivity, difficulty in interpreting the obtained spectra, and presence of analytes deficient in protons or which possess protons that can be readily interchanged with the solvent (Clarke and Haselden 2008; Villas-Boas et al. 2005).

In MS, the analytes present in the sample are ionized and characterized by their mass-to-charge ratio (m/z). MS detection is usually preceded by a separation technique that aims to resolve the individual components present in complex biological

Table 1 Summary of advantages and limitations of MS and NMR techniques

Platform	Advantages	Disadvantages
NMR	Quantitative Nondestructive High throughput Requires minimal sample preparation Robust, highly reproducible technology Compatible with liquids and solids	Low sensitivity (μM range) Low resolution Complex data processing Requires large sample volume/quantity Limited to protonated compounds
MS	High versatility, has the potential of covering a wide part of the metabolome Can be hyphenated to previous separation techniques (i.e., GC, LC, and CE) High sensitivity (especially in the case of LC-MS, nM-pM range) High resolution and selectivity (when needed/desired) Possibility of targeted and untargeted metabolic profiling modes Usually requires low sample volume/quantity	Destructive Limited reproducibility (mainly when hyphenated to LC and CE) Usually requires extensive sample preparation Long analysis times when hyphenated to previous separation techniques Quantitation highly dependent on calibration curves and appropriate internal standards and chemical reference compounds

matrices. The most widely used separation techniques coupled to MS are GC, liquid chromatography (LC), and capillary electrophoresis (CE). GC is used to separate volatile (and nonvolatile, after derivatisation) metabolites (Dunn 2008; Lenz and Wilson 2007). CE separates polar ionisable compounds based on their *m/z* (Ibanez et al. 2012). LC is by far the most widely used separation technique in metabolomics and allows the separation of metabolites based on their chemical properties according to the stationary phase of the selected chromatographic column (Lenz and Wilson 2007; Dettmer et al. 2007). Traditional LC separations have been performed by reversed phase (RP) chromatography (Lenz and Wilson 2007). RP-LC is usually performed with C18-bonded silicas as stationary phases and a water to methanol or acetonitrile gradient. RP-LC is suitable for retaining and separating medium-polar and nonpolar metabolites and is a good option as a starting point in metabolomic studies. However, very polar compounds elute in the void volume or with minimal retention. Hydrophilic interaction chromatography (HILIC) and ion-pairing liquid chromatography (IP-LC) are two alternative strategies for the separation of metabolites that are poorly retained in RP. IP-LC is based on the use of ion-pair modifiers, large ionic molecules that have both a hydrophobic region that interacts with the stationary phase and a charged region that interacts with the analyte, while maintaining the typical water to methanol/acetonitrile gradient of RP-LC (Cajka and Fiehn 2016). The stationary phases used in HILIC chromatography include amine, amide, or free silanol groups, and, unlike RP, separation starts with a high proportion of organic solvent, while water is considered a highly eluotropic solvent. Metabolite retention is a combination of liquid-liquid

partitioning, adsorption, ionic interactions, and hydrophobic retention and heavily depends on the nature of the analyte and the composition of the mobile phase (Buszewski and Noga 2012). No single separation technique is able to resolve and detect the complete range of metabolites that may be present in a complex biological sample. Therefore, achieving the most comprehensive metabolome coverage may require the use of several column chemistries, or even complementary separation techniques (Dettmer et al. 2007; Leon et al. 2013). The main advantages of MS hyphenated to separation techniques are: (i) its high sensitivity, several orders of magnitude lower than NMR; (ii) its high resolution and selectivity; (iii) the possibility of performing fragmentation analyses to thus confirm the identity of the detected metabolites and the identification of unknown and unexpected compounds; and (iv) information is easier to handle than in NMR spectroscopy (Leon et al. 2013; Villas-Boas et al. 2005). In addition, MS allows analyses not only in a holistic or global way (untargeted or non-directed analyses), where the aim is to analyze the largest possible number of compounds, but it also enables guided analyses (targeted), in which detection focuses on a specific group of metabolites of interest, which enables work to be done with increased sensitivity and quantitatively (Robertson 2005). Although the latter may not be considered a true "omics" approach because it basically consists in a biased analysis, which is usually driven by a hypothesis or by a priori knowledge of the system under study, targeted analyses may be considered an important part of untargeted metabolomics. Once potential biomarkers have been deciphered by untargeted metabolomics analyses, they should be validated by performing targeted quantitative analyses (Fig. 3; Leon et al. 2013). Regarding MS detection, untargeted metabolomics requires instruments with a sensitive full-scan mode and accurate mass measurement. Quadrupole time of flight (Q-TOF) mass spectrometer meets such requirements as it combines the stability of a quadrupole with the high efficiency, sensitivity, and accuracy (<5 ppm) of a TOF. It also offers mass fragmentation capabilities for metabolite identification (Lahoz et al. 2006). Thanks to its capabilities, the Q-TOF mass spectrometer has become the instrument of choice for untargeted analysis approaches, although other instruments, such as Orbitrap or Fourier Transform Ion Cyclotron Resonance (FT-ICR), also meet high mass resolution capabilities (Leon et al. 2013). Targeted metabolomics requires the unambiguous identification and quantification of metabolites of interest. Triple quadrupole (TQ) mass spectrometers, which mostly work in the multiple reaction monitoring (MRM) mode, are the most common platform to perform targeted analyses. TQ mass spectrometers have a lower mass resolution than Q-TOF but offer greater sensitivity, specificity, robustness, and a wider dynamic range (Leon et al. 2013). Given that LC-MS is the most commonly used platform for holistic metabolome analysis, the following subsections of the present section are detailed for this particular case, although some steps can be extended to other analytical platforms.

Quality Control Analysis

The main premise in a metabolomics study is that the levels of detected metabolites reflect the biological status of the system under study. So besides the careful selection and definition of the abovementioned factors (i.e., type of sample, sampling

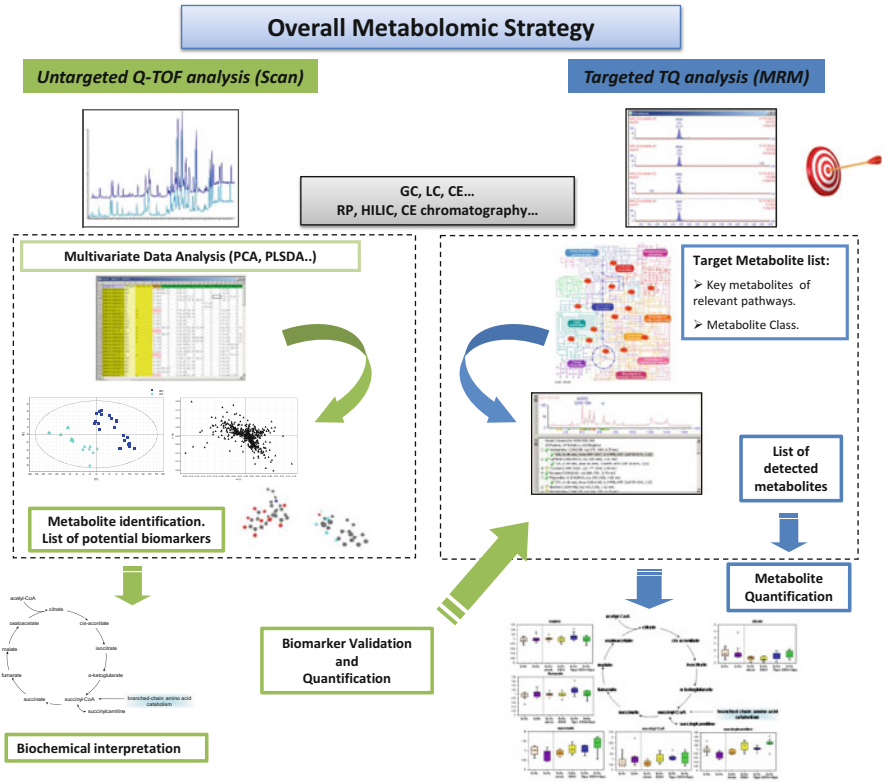

Fig. 3 Global MS-based metabolomics strategy. An initial assessment is made by using unbiased untargeted analyses to obtain a broad view of the metabolome to search for potential biomarkers. In a second step, a targeted approach is performed to provide an unambiguous quantitative assessment of the metabolites of interest

conditions, sample processing, analytical conditions to perform the metabolome analysis, etc.), it is mandatory to implement a quality assurance strategy during sample preparation and analyses to ensure the quality of the results and to also minimize and detect any sources of variation unrelated to the biological nature of the samples (Garcia-Cañaveras et al. 2011; Quintás et al. 2012). As real method validation is hardly achievable in untargeted metabolomics (Naz et al. 2014), quality assurance is usually based on the addition of internal standards to samples, the inclusion of quality control (QC) samples (i.e., blanks, pooled samples, commercially available pools, etc.), and the careful design of the sample acquisition process (Garcia-Cañaveras et al. 2011; Naz et al. 2014).

Metabolomic Data Analysis

Holistic (untargeted) LC-MS-based metabolomics analyses generate huge amounts of data. The information obtained for each sample is arranged into a great three-

dimensional matrix (retention time (rt), *m/z*, and intensity), in which each feature is informative of a metabolite, fragment, or adduct present in the sample. Before performing statistical analyses, it is necessary to process metabolomics data. Among other steps, processing includes the alignment of chromatographic peaks among samples and the standardization of variables to enable inter-sample comparisons. These processes can be performed by vendor's software (e.g., Markerlynx, Markerview, Masshunter, ProGenesis), by open access software (e.g., XCMS, MZmine), or by in-house scripts (e.g., R, Matlab) (Leon et al. 2013). Processed information can then be subjected to both uni- and multivariate analyses, although the latter is preferred given the nature of the data (Fig. 4). Regarding multivariate data analyses, two types of methods can be adopted: non-supervised (e.g., Principal Components Analysis (PCA)) and supervised methods (e.g., Partial Least Squares Discriminant Analysis (PLS-DA) or orthogonal projection to latent structures-discriminant analysis (OPLS-DA). In both analyses, the vast quantity of the experimental variables obtained (mega matrix) is reduced to a small number of latent variables (principal components) that explain in a much simpler way the similarities or differences between the samples (observations) and variables (metabolites) responsible for these differences or similarities. In a PCA, the first principal component describes the main difference or variance between samples, the second principal component (independent and orthogonal) describes the residual variance of samples after that explained by the first component, and so on. The main difference between supervised and non-supervised methods is that the algorithm in the latter relies on previous knowledge to, for example, classify samples (observations). So it finds the latent variable that best describes the differences between samples by taking into account which group they belong to (Robertson 2005; Kaddurah-Daouk et al. 2008). As in data preprocessing, data analyses can be performed by specific software (i.e., MassProfiler Professional, SIMCA, etc.), with freely available packages or in-house built scripts that use the R software (R Core Team 2014), or even with user-friendly guided tools, as is the case of the MetaboAnalyst (Xia et al. 2015), which is a set of online tools for metabolomic data analysis and interpretation.

Metabolite Identification

The last step in a metabolomic analysis is to identify the potential biomarkers selected in the multivariate analysis (Fig. 4). According to Metabolomics Standards Initiative criteria (Sumner et al. 2007), four levels of metabolite identifications can be found in the published metabolomics literature: (1) identified compounds: identity has been corroborated by the analysis of the authentic standard under the same analytical conditions and parameters, such as retention time (rt), and MS and mass fragmentation (MS/MS) spectra can be matched; (2) putatively annotated compounds (e.g., without chemical reference standards, based on physicochemical properties and/or spectral similarity with public/commercial spectral libraries); (3) putatively characterized compound classes (e.g., based on the characteristic physicochemical properties of a chemical class of compounds or by spectral similarity to known compounds of a chemical class); (4) unknown compounds: although

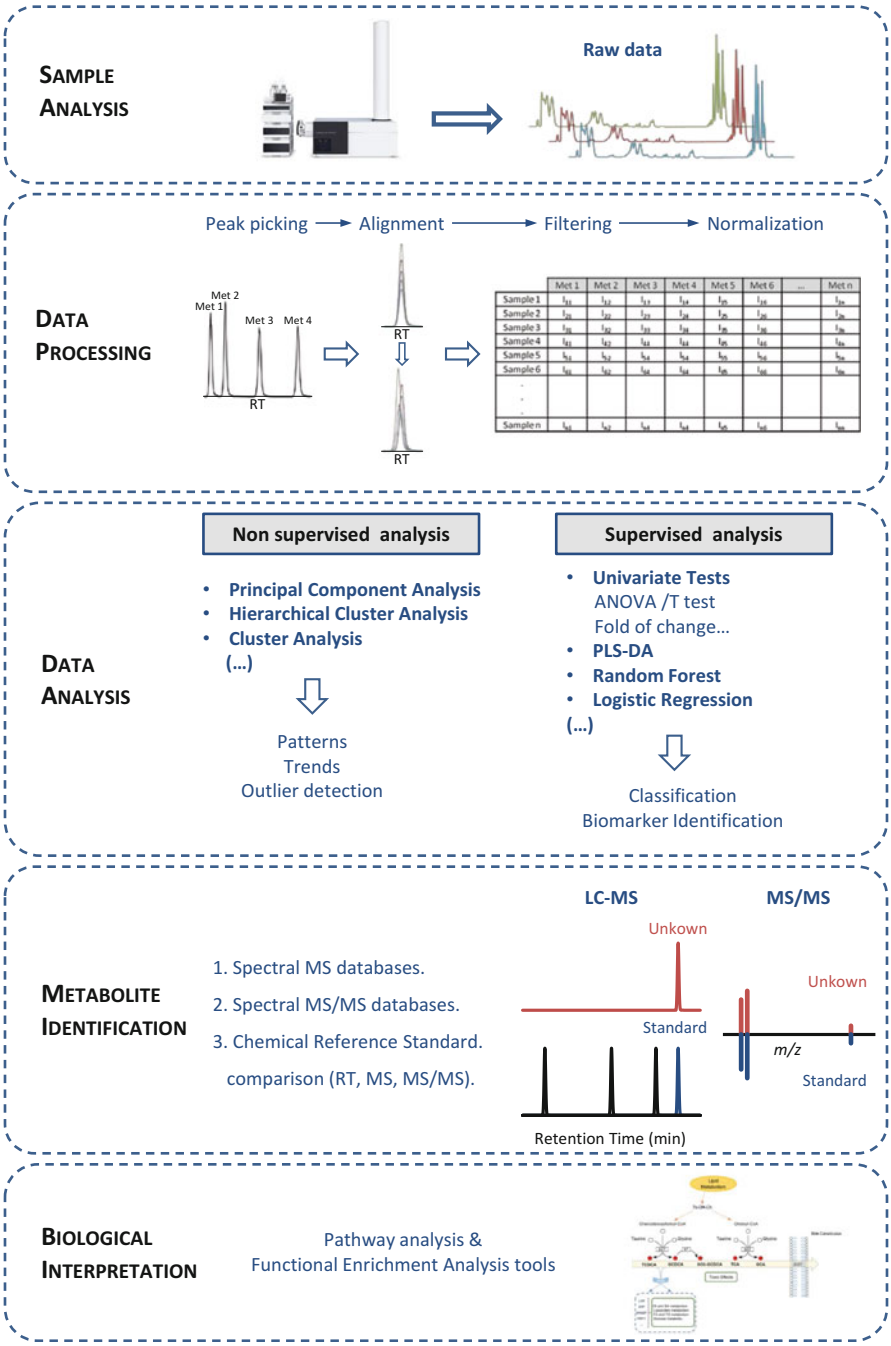

Fig. 4 The data workflow in MS-based metabolomics analyses. Several steps are needed before raw data can be interpreted and placed in a biological context. The first data workflow step is termed data processing and may include different steps, such as peak peaking, peak alignment, filtering, and data normalization or transformation. In the end, the raw data generated during sample analyses

unidentified or unclassified, these metabolites can still be differentiated and quantified according to spectral data (Sumner et al. 2007). While the definitive assignment of a feature (characterized by rt and *m/z*) to a known identity requires the use of chemical reference standards, initial metabolite identification stages are usually performed based on the use of publicly or commercially available databases. Some useful available online metabolite databases that allow to search both MS and MS/MS data are the Human Metabolome Database (Wishart et al. 2013), the LIPID MAPS-Nature Lipidomics Gateway (Fahy et al. 2007), the Metlin Database (Smith et al. 2005), and MassBank (Horai et al. 2010).

Biological Interpretation

After identifying the metabolites that are significantly altered as a result of a given pathophysiological situation of interest, it is of special relevance to place them in a context to extract useful and meaningful information (Fig. 4). Online pathway analysis tools, such as MBRole 2.0 (López-Ibáñez et al. 2016), metabolite set enrichment analysis, and metabolic pathway analysis, for metabolomic data interpretation integrated into MetaboAnalyst (Xia et al. 2015), may be helpful for this purpose.

Metabolomics in Organ Transplantation

Metabolomics/metabolic profiling, either MS or NMR based, has been used in many human health (or biomedicine) areas and covers a wide spectra of matrices from cells in culture (IPS, hepatocytes, cell lines, etc.) (Garcia-Cañaveras et al. 2016) to body fluids (urine, serum, bile, cerebrospinal fluid, etc.) (Soga et al. 2006; Trushina et al. 2013; Garcia-Cañaveras et al. 2012) and even tissues (liver, tumors, heart, adipose tissue, etc.) (Garcia-Cañaveras et al. 2011; Chan et al. 2009) and in a high diversity of study fields (cancer, cardiovascular disease, nonalcoholic fatty liver disease, diabetes, toxicity, etc.) (Brindle et al. 2002; Puri et al. 2007; Rhee et al. 2011; Cortes et al. 2014; Dang et al. 2009; García-Cañaveras et al. 2015). The success in such a diversity of biological samples and fields of application reflects the potential that metabolomics has to be really and fully incorporated into the clinical field.

Metabolomics has become an extremely useful tool to characterize the metabolic changes that can take place in an organ. Application of metabolomics in transplantation is still in its early stages, but metabolomic studies in solid organ transplants

Fig. 4 (continued) is arranged in a mega matrix that contains all the information. Then data analysis procedures are applied, which include both supervised and non-supervised methods and also uni- and multivariate techniques. Those features were found to be relevant according to the aim of the data analysis and are then subjected to metabolite identification procedures (various grades of identification confidence can be achieved based on the criteria established by the Metabolomics Standards Initiative). Finally, biological interpretation can be simplified by using freely available web-based tools to perform pathways analyses and/or functional enrichment analyses

have generally focused on monitoring three situations: (i) post-reperfusion damage, (ii) rejection, and (iii) organ dysfunction (Wishart 2008). Of all the material published on the matter, 60% is about renal transplants, followed by the liver (21%), heart (10%), pancreas (5%), and lungs (6%). Most metabolite measurements for organ transplantation have been performed ex vivo using body fluids like urine, serum, or bile (Sinclair et al. 1974; Saude et al. 2004; Hauet et al. 2000; Serkova et al. 2005; Silva et al. 2005; Gibelin et al. 2000; Martin-Sanz et al. 2003). Examples in organ transplantation include the diagnosis of acute cardiac rejection by analyzing plasma by ^{1}H-NMR spectroscopy (Mouly-Bandini et al. 2000), profiling acute renal rejection by GC-MS (Mao et al. 2008), and monitoring kidney transplant patients' immune responses and drug effects in early recovery by means of urine samples analyzed by ^{1}H-NMR (Stenlund et al. 2009). Most measured molecules are related to metabolic processes that generally exist in any living being, such as glycolysis, gluconeogenesis, lipid metabolism, etc. Changes in universal metabolites, such as glucose, citrate, lactate, ATP, and AFP reflect changes in cell viability, like apoptosis, levels of oxygenation (anoxia, blood flow), local pH, and homeostasis in general (Saude et al. 2004). These molecules provide information about cell function or cell stress and, therefore, about organ function. Other less used metabolites, like thromboxane, histamine, or chlorotyrosine, could reflect the immune function of inflammatory response (Sinclair et al. 1974; Saude et al. 2004).

Liver Transplantation

The use of metabolomics in liver transplantation (LT) is a challenge as it deals with an organ involved in many metabolic processes. Such a complex scenario hinders the possibility of finding a single biochemical test to generally assess liver function (Sakka 2007). The therapeutic success of LT has meant that more patients are susceptible to taking advantage of it. This increased demand has generated longer waiting lists, plus an increase in morbidity and mortality. In view of this situation, and in order to increase the number of donors, the criteria which define whether an organ is suitable or not have gradually changed, which means that "marginal" or "extended criteria" livers are being used with the subsequent risk of postoperative complications appearing, such as severe graft dysfunction and primary liver failure (Vilca Melendez et al. 2000). The open questions are: To what extent can we expand donor criteria? Which criteria should we apply to make the decision as to whether a liver can be used or not? And, in this context, can metabolomics provide valuable information? Despite the fact that organs from extended criteria donors (ECD) are not optimal, they are a good alternative to dying while being on a transplant waiting list (Busuttil and Tanaka 2003). Many factors play an important role in the onset of graft dysfunction or primary failure (Chen et al. 2007). Damage caused by ischemia/reperfusion injury (IRI) could be responsible for graft dysfunction in many cases. Hypothermia lowers the metabolism and helps maintain essential metabolic functions but induces cell damage (alterations in calcium homeostasis, cytoskeleton modifications, and local tissue destruction by proteases). Reperfusion implies the

production of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radicals, all of which are involved in IRI (Silva et al. 2007). Factors like pH, inflammatory response, and microcirculation changes aggravate cell damage. Despite their importance, all the mechanisms that cause graft dysfunction are not yet completely known.

During the first week after transplantation, most grafts show some sign of liver dysfunction, 20% of which are attributed to a defect in the liver's metabolic capacity. Some of the factors responsible for this should be considered *a priori* as potentially controllable, and they include the metabolic and functional quality of the graft. The availability of an objective criterion to assess graft quality before implant would be extremely useful for making decisions that would minimize the risk of severe metabolic dysfunction and/or primary liver failure. If this type of information could be made available together with the usual provided information, such as an anatomopathological study, it could avoid discarding an organ simply because of its macroscopic appearance (surgeon-related subjective criterion that does not always coincide), or elusive histology, and would therefore increase the number of useable organs based on objective criteria selection. Several studies have been carried out about LT using NMR and MS to quantify the graft injury secondary to cold preservation (Silva et al. 2007; Gibelin et al. 2000), graft recovery following transplant (Silva et al. 2007), and to also identify diagnostic and prognostic biomarkers of graft rejection and dysfunction (Martin-Sanz et al. 2003; Singh et al. 2003; Melendez et al. 2001; Cortes et al. 2014; Table 2). More detailed information about the data published on metabolomics, IRI, and graft function in LT is provided.

Ischemia Reperfusion Injury

During LT, a donor graft initially undergoes a period of ischemia from the time it is retrieved from the donor until blood supply is restored on reperfusion in the recipient, which enhances any damage produced during the ischemic period. This situation is termed IRI, which can result in poor graft function after transplantation (Serracino-Inglott et al. 2001). In 2003, *Martin-Sanz et al.* demonstrated decreased nitric oxide (NO) synthesis as a result of higher rates of nitric oxide synthase (NOS) inhibition in blood samples before reperfusion in recipients who presented graft dysfunction after LT. They indicated that lower NO levels can cause ischemia related to vasoconstriction and can participate in IRI (Martin-Sanz et al. 2003). A potent NOS synthase named asymmetric dimethylarginine (ADMA) was found in these patients, which showed increased levels in parallel to cold ischemia time (CIT) duration.

High lactate levels are usually found in ischemic processes where cells start anaerobic glycolysis, which results in raised lactate and lower pyruvate levels (Sommer and Larsen 2004). *Silva et al*. observed high levels of lactate and pyruvate upon reperfusion, which slowly normalized during the following 12 h if the liver recovered from initial ischemic insult. These authors also described that the levels of four amino acids (alanine, GABA, glutamate, and taurine) lowered during the monitoring period, but at different rates and time points (Silva et al. 2007).

Table 2 Metabolic markers associated with ischemia reperfusion injury and graft dysfunction in humans

Author	Condition	Increased metabolites	Platform	Decreased metabolites	Samples
Vilca-Melendez 2001	Posttransplant	Phosphatidylcholine	^{1}H-NMR		Bile
Martin-Sanz et al. 2003	Ischemia reperfusion injury	Methylarginine dimethylarginine	HPLC-MS		Perfusate Blood
Singh et al. 2003	Graft dysfunction	Glutamine	^{1}H-NMR	Urea (urine)	Blood Urine
Silva 2005	Ischemia reperfusion injury	Lactate, pyruvate, glycerol, alanine, glutamate, GABA, taurine, arginine (<19 h)	HPLC-MS CEAD	Arginine (<19 h) Synthesis of NO	Dialysate
Serkova 2007	Graft dysfunction	Lactate, uric acid, and citrate	^{1}H-NMR		Blood
Tripathi et al. 2009	Graft dysfunction	Lactate, alanine, lysine, glutamine, methionine, asparagine, histidine, tyrosine, and phenylalanine	^{1}H-NMR		Blood (serum)
Hrydziuszko et al. 2010	Ischemia reperfusion injury	Urea and urea cycle intermediate levels (e.g., N4-acetylaminobutanal, 5'-methylthioadenosine) Increased bile acid levels (e.g., chenodeoxyglycocholate, glycodeoxycholate, glycochenodeoxycholate, and glycholate) Disturbance of energy metabolism (formate, orthophosphate, ADP, fumarate, succinate)	FT-ICR MS CEAD		Liver tissue Dialysate
Cortes et al. 2014	Graft dysfunction	Lysophosphatidylcholines and lysophosphatidylethanolamines, phosphatidylcholines, phosphatidylethanolamines, sphingomyelins, bile acids, and products of histamine metabolism	UPLC-MS		Liver tissue

In 2009, *Hrydziuszko et al*. compared the metabolic profile of liver biopsies after both procurement and reperfusion and used a FT-ICR MS-based metabolomics approach with colorimetric electrochemical array detection (CEAD) in microdialysates for the first time. The main metabolic changes observed upon reperfusion were increased urea production, higher urea cycle intermediate levels (e.g., *N*4-acetylaminobutanal, 5-methylthioadenosine), and raised bile acid levels (e.g., chenodeoxyglycocholate, glycodeoxycholate, glycochenodeoxycholate, and glycholate). Further molecular changes included the anticipated disturbance of energy metabolism, with consistent increases in several metabolites (e.g., formate, orthophosphate, ADP), particularly those involved in oxidative phosphorylation (e.g., fumarate, succinate) in post-reperfusion biopsies. One of these authors' major findings was that the metabolite profile in a donor after circulatory death (DCD) in the cold phase was similar to the metabolic profile after reperfusion in those livers obtained from donors after brain dead (DBD) (Hrydziuszko et al. 2009).

Graft Function Assessments

Extended criteria donors in LT (elderly donors, DCD, etc.) are increasingly becoming a source of organs, which are more susceptible to ischemic insult. IRI plays a central role in posttransplant complications, especially in graft function. Therefore, the expansion of donor criteria requires an objective quality graft assessment to predict or avoid complications (Vogel et al. 2012). Many tests have been evaluated to assess the pretransplant graft function. To date, however, none has found its place in the clinical practice (Vilca Melendez et al. 2000). Bile secretion has been generally accepted as an early posttransplantation sign of liver recovery (Ericzon et al. 1990). However, bile secretion was not studied in donor livers until 1998, when *Vilca-Melendez et al*. focused on analyzing its profiling (bile acid composition) in donors upon organ retrieval, and in recipients immediately after reperfusion, after developing a standardized bile collection technique (Vilca-Melendez et al. 1998). This study showed no difference in bile flow to differentiate between "suboptimal" and normal grafts. However, these "suboptimal" grafts showed a higher concentration of bile acids, which indicated that bile flow did not increase appropriately with the higher concentration of bile acids. The same authors postulated that this finding could be related to water secretion impairment at a canalicular level or due to a reduction in the bile acid-independent promoters of bile flow, such as glutathione, bicarbonate, calcium, sodium, potassium, glucose, amino acids, and organic acids. The donor bile from suboptimal grafts had a higher proportion of cholic acid than normal grafts. It is well known that the canalicular bile flow depends not only on the amount of bile acids secreted but also on bile acid composition (Howard and Murphy 1990).

An impairment in the urea cycle during acute liver failure results in abnormally high levels of blood ammonia, which triggers glutamine synthesis (Suarez et al. 2002) and decreased urea levels. *Sing et al*. observed higher glutamine levels in blood and urine, and lower urea levels in urine, by ^{1}H-NMR spectroscopy in a patient who presented liver failure related to vascular complications after transplantation. Thus monitoring glutamine levels in blood and urine, along with urea levels in urine, has been proposed as a predictor of graft function (Singh et al. 2003).

Increased circulating amino acids (e.g., tyrosine, glutamine, leucine) have been correlated with decreased catabolism by the liver, which reflects hepatocyte injury and death (Saxena et al. 2006). These results were confirmed by *Tripathi et al*., who analyzed serum specimens by ^{1}H-NMR in liver transplant patients preoperatively and at various time points following transplantation. These authors observed high levels of lactate, alanine, lysine, glutamine, methionine, asparagine, histidine, tyrosine, and phenylalanine in the patient who died after LT due to graft dysfunction (Tripathi et al. 2009). This finding agrees with the earlier analyses performed both on patients with experimental models of chronic liver failure (Tietge et al. 2002).

Duarte et al. analyzed liver biopsies, which were collected at three different time points during LT: before organ retrieval, during cold storage, and after implantation. The purpose was to find metabolic signatures that reflected graft success. The metabolomic platform used was high-resolution magic angle spinning (HR-MAS) ^{1}H-NMR, a variation of conventional ^{1}H-NMR that can be performed on solid samples, and it was the first metabolomic application of such spectroscopy used with human liver tissues. A larger amount of triglycerides and unsaturated lipids, and lower levels of phospholipids, were found on the grafts with fatty infiltration (Duarte et al. 2005). Recently, *Xu et al*. described for the first time different lipid profiles between two types of donors as DBD and DCD. For this purpose, they initially performed an untargeted approach, followed by a targeted analysis using UPLC-MS. DCD livers showed higher concentrations of LysoPCs, which is a known precursor of the platelet-activating factor, a potent phospholipid inflammatory associated with IRI (Xu et al. 2015).

Despite all the potential biomarkers described in the literature, transplant surgeons still have to rely on subjective donor data interpretations, evaluations of the macroscopic appearance of the graft (shape, color, appearance, and feel), and occasionally on the histological analysis of a liver biopsy, in order to assess the graft's suitability for use. Liver biopsies assess the degree of steatosis, fibrosis, sepsis, and ischemia. Yet some controversy still exists when evaluating organs that present mild to moderate steatosis. What this reflects is both the difficulty to predict graft functionality based on changes in morphology (Angele et al. 2008) and lack of a functional assessment that really helps rule out grafts with a high risk of primary non-function, or to accept organs that, based on subjective assessments, would have been ruled out for transplant. Recently, a metabolomics attempt to predict donor liver function after transplantation has proven to be a useful tool to assess organs before transplantation. In this work, a metabolomic pattern, which allows donor quality assessment, has been deciphered by using a MS-based metabolomic approach to analyze the liver biopsies collected after organ retrieval (Cortes et al. 2014). The novelty of this approach lies in the use of two chromatographic techniques (i.e., RP and HILIC), which allow the coverage of the metabolites identified in the previous LC-MS-based analysis to be extended (Cortes et al. 2010). Using multivariate data analysis (i.e., PCA and PLS-DA), the relationship between the metabolomic profile present in liver biopsies and their subsequent function in the

recipient according to the Olthoff classification (Olthoff et al. 2010) was investigated. A set of 93 metabolites was used, which are significantly involved in the metabolic processes related to early allograft dysfunction (EAD) and initial good function (IGF) distinctions, and are made up of amino acids and peptides, carbohydrates, vitamins and cofactors, bile acids, carnitines, fatty acids, products of the glycerolipid metabolism, lysophospholipids, phospholipids, and sphingomyelins. The patients who presented EAD showed significantly higher levels of lysophosphatidylcholines and lysophosphatidylethanolamines, lysophospholipids, phosphatidylcholines, phosphatidylethanolamine, phospholipids, sphingomyelins, bile acids, and products of histamine metabolism (Fig. 5). The lipidomic pattern found in this study could prove to be an interesting diagnostic instrument in clinical practice as phospholipid homeostasis alteration may indicate cellular membrane disruption, which would thus trigger different mechanisms of hepatocellular death (Arora et al. 1997). It has been recently demonstrated that lysophosphatidylcholines are toxic metabolites generated by the hydrolysis of the phospholipids catalyzed by phospholipase A2, which acts as a promoter of cell death (Kakisaka et al. 2012; Han et al. 2008). The significant accumulation of lysophosphatidylcholines observed in the EAD group suggests a greater predisposition of these grafts to lipid-dependent apoptosis, which can dramatically affect posttransplant graft functioning. Bile salts are considered key signal molecules as physiological ligands of the farnesoid X receptor, an intracellular sensor that controls the expression of the genes involved in the metabolism of lipids, lipoproteins, and glucose (Hylemon et al. 2009). Previous publications have suggested that bile salts are powerful function markers used to monitor LT and rejection (Vilca Melendez H et al. 2004). In agreement with the findings described by *Vilca-Melendez et al*., the excessive accumulation of bile salts and phospholipids observed in poor-quality liver grafts could make the initial bile excretion in the liver graft difficult, once it has been implanted, which is considered an early sign of graft function (Vilca Melendez et al. 2004; Hedaya et al. 2009). The key role of homeostasis in bile salts during progression after LT is supported by previous studies that have reported the altered expression of numerous genes related to the synthesis of bile acids and their transport (e.g., BAAT, CYP7A1, SULTA2, MDR2, BSEP), as well as the nuclear factors involved in the regulation of these genes (e.g.,: HNFα, FXR, SREBF1) associated with early graft dysfunction (Fouassier et al. 2007; Defamie et al. 2008). In grafts that present EAD, alterations have been found in other metabolic pathways that are not directly related to lipid metabolisms, such as histidine metabolism. High histamine levels could be indicative of the adaptive response to liver damage as a result of CIT or graft preservation for the purpose of reducing the release of cytokines thorough histamine H4 receptor activation (Motoki et al. 2008). It has also been proven that histamine acts via receptors H1-H4 to trigger the signal for metabolic pathways by proliferating and differentiating from cholangiocytes. These regulating processes have to be forced under cholestatic conditions, like those previously described in grafts with EAD (Francis et al. 2012).

Pathway	In bckgnd.	hits	*p-value*	*Adj. p-value*
Phospholipid Biosynthesis	19	6	2.70E-05	5.64E-04
Bile Acid Biosynthesis	49	5	1.85E-02	6.78E-02
Histidine Metabolism	11	4	4.90E-04	3.04E-03
Ammonia Recycling	18	4	4.46E-04	3.16E-03
Aminoacyl-tRNA biosynthesis	28	3	2.06E-02	6.78E-02
Urea Cycle	20	2	1.37E-02	1.14E-02
Glycerolipid Metabolism	13	2	3.08E-02	8.85E-02
Glycerol Phosphate Shuttle	8	2	1.18E-02	6.78E-02
Glutathione Metabolism	10	2	1.85E-02	6.78E-02

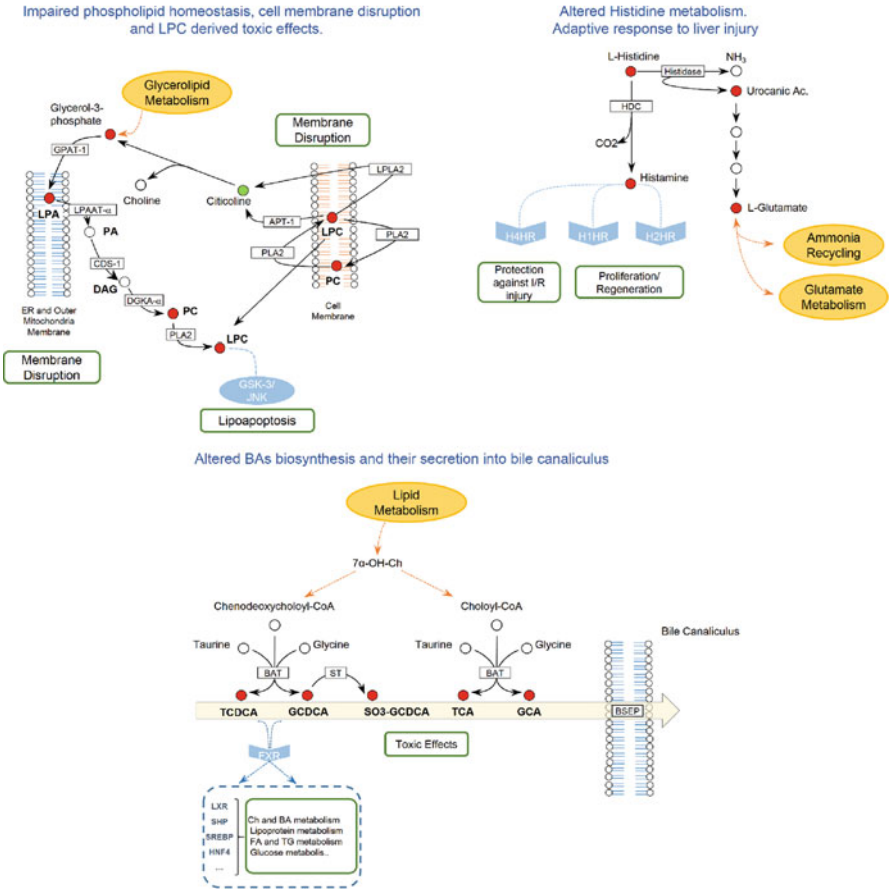

Fig. 5 Biological interpretation of the EAD metabolomic biosignature. (**a**) Summary panel for the metabolite set enrichment analysis where metabolic pathways are ranked according to their impact on EAD. (**b**) Impaired phospholipid homeostasis derives in cell membrane disruption and provokes toxic effects, triggered by lysophosphatidylcholine (LPC) accumulation. (**c**) Altered lipid metabolism provokes downstream alterations of bile acid biosynthesis and accumulation derives into toxic effects and FXR receptor activation. (**d**) Alteration in histidine metabolism as a response to ischemia. The metabolites depicted in green and red are, respectively, down- or upregulated

Future Applications of Metabolomics in Liver Transplantation

The increasing number of patients on waiting lists for LT has driven transplant centers to use ECD, which has raised the posttransplantation incidence of EAD up to 27%. This condition is associated with increased rates of suffering acute cellular early rejection, sepsis, and with longer intensive care unit and hospital stays, which can result in higher rates of graft loss, recipient morbidity, and mortality (Briceño and Ciria 2010; Salvalaggio et al. 2013). As previously mentioned, many efforts have been made to assess graft function before transplant by evaluating different liver metabolism aspects: bile secretion, hepatic protein synthesis, drug-metabolizing capabilities, organ morphology, etc. (Vilca-Melendez et al. 2000). However, a well-accepted functional assessment of an organ before its transplantation is still lacking. Therefore, the discovery of objective biomarkers that can assess graft quality and anticipate its later function would be greatly appreciated and be of much interest, especially when evaluating ECD. In addition, the need to increase the donors' pool has led to an increased utilization of DCD grafts, which itself is considered an extended criteria that has been associated with higher early graft failure and cholangiopathy rates (Garcia-Valdecasas et al. 1999; Kukan and Haddad 2001). While such grafts are being assessed, it is difficult to evaluate the liver damage caused, particularly, by warm ischemia time, which cannot be observed macroscopically. Therefore, markers that could predict the posttransplant graft function would constitute an invaluable tool to help decide whether to accept it or not.

Normothermic machine perfusion (NMP) has become an emerging preservation modality designed to maintain the liver metabolically active during storage and has the potential to prevent injury associated with low temperature and to promote physiological organ repair following ischemic cell damage. Several animal studies have demonstrated the feasibility and superiority of normothermic liver perfusion during cold storage (CS) with a lower inflammatory response after reperfusion and longer survival rates (Fondevila et al. 2011). The benefits of this technique are that it allows the thorough analysis of its quality by measuring parameters, e.g., bile production, lactate, glucose, and oxygen composition. In this context, the metabolomic analysis of bile and/or the perfusate obtained from the graft would add not only the liver quality assessment but also the effect of normothermic preservation on the graft. More importantly, this metabolomic analysis would become a priceless tool during organ assessment to recover livers that have been previously discarded when preserved in cold.

Although the use of metabolomics in organ transplantation is in its early stages, the above-described applications indicate its huge potential in this field. Assessing donor liver quality before LT in order to make the most of limited resources available, and to anticipate possible clinical complications related to graft function, remains a challenge. Furthermore, a rigorous validation of the metabolomic procedures in multicentre studies is mandatory to ensure their usefulness in a clinical environment.

Summary Points

- The term biomarker refers mostly to macromolecules or metabolites which have become surrogate markers in basic and clinical research and in clinical practice and offer the advantage of being an objective, quantifiable, and reproducible measure.
- Biomarkers offer different applications in the human health field, not only once the disease is present but also to predict its potential appearance.
- Metabolomics, through the simultaneous measurement of hundreds of small molecules, can provide a more comprehensive "snapshot" than the simple determination of a single marker.
- Metabolomic studies in solid organ transplants have focused especially on monitoring three situations: post-reperfusion damage, rejection, and dysfunction of the organ.
- Markers of graft dysfunction and ischemia reperfusion damage indicate the following metabolic pathways/function: amino acids, urea cycle, bile acids, and energy metabolism.
- The most novel use of metabolomics in liver transplantation is the assessment of donor liver quality before transplantation.
- The following pathways/groups of metabolites have been found to be markers of organ quality, determined based on the function of the graft once transplanted: bile acids, histamine metabolism, phospholipids, and lysophospholipids.
- Metabolomics has the potential to be a crucial tool in assessments of graft quality before liver transplantation, in anticipating clinical complications related to graft function and in assessing the parameters that affect graft quality during its manipulation before implantation.

Acknowledgments This work has been supported by the European Regional Development Fund, the Carlos III Health Institute of the Spanish Ministry of Economy and Competitiveness (PI14/0026), and by the Roche Organ Transplantation Research Foundation. A. L. is grateful for a Miguel Servet II contract (CPII14/0004) from the above Ministry/Carlos III Health Institute.

References

Angele MK, Rentsch M, Harlt WH, et al. Effect of steatosis on liver function and organ survival after liver transplantation. Am J Surg. 2008;195(2):214–20.

Arora AS, Jones BJ, Patel TC, et al. Ceramide induces hepatocyte cell death through disruption of mitochondrial function in the rat. Hepatology. 1997;25:958–63.

Bairaktari E, Katopodis K, Siamopoulos KC, et al. Paraquat-induced renal injury studied by 1H NMR spectroscopy of urine. Clin Chem. 1998;44:1256–61.

Beckwith-Hall BM, Nicholson JK, Nicholls A, et al. Nuclear magnetic resonance spectroscopic and principal components analysis investigations into biochemical effects of three model hepatotoxins. Chem Res Toxicol. 1998;11:260–72.

Beecher CW. The human metabolome. In: Garrigan GG, Goodacre R, editors. Metabolic profiling: its role in biomarker discovery and gene function analysis. Springer; 2003. p. 1–8.

Biomarkers Definition Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001;69:89–95.
Bory C, Boulieu R, Chantin C, et al. Diagnosis of alcaptonuria: rapid analysis of homogentisic acid by HPLC. Clin Chim Acta. 1990;189:7–11.
Bouatra S, Aziat F, Mandal R, et al. The human urine metabolome. PLoS One. 2013;8:e73076.
Briceno J, Ciria R. Early graft dysfunction after liver transplantation. Transplant Proc. 2010;42:631–3.
Brindle JT, Antti H, Holmes E, et al. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using 1H-NMR-based metabolomics. Nat Med. 2002;8:1439–44.
Busuttil RW, Tanaka K. The utility of marginal donors in liver transplantation. Liver Transpl. 2003;9:651–63.
Buszewski B, Noga S. Hydrophilic interaction liquid chromatography (HILIC) – a powerful separation technique. Anal Bioanal Chem. 2012;402:231–47.
Cajka T, Fiehn O. Toward merging untargeted and targeted methods in mass spectrometry-based metabolomics and lipidomics. Anal Chem. 2016;88:524–45.
Chagoyen M, Pazos F. MBRole: enrichment analysis of metabolomic data. Bioinformatics. 2011;27:730–1.
Chan EC, Koh PK, Mal M, et al. Metabolic profiling of human colorectal cancer using high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy and gas chromatography mass spectrometry (GC/MS). J Proteome Res. 2009;8:352–61.
Chen H, Peng CH, Shen BY, et al. Multi-factor analysis of initial poor graft function after orthotopic liver transplantation. Hepatobiliary Pancreat Dis Int. 2007;6:141–6.
Clarke CJ, Haselden JN. Metabolic profiling as a tool for understanding mechanisms of toxicity. Toxicol Pathol. 2008;36:140–7.
Cortes M, Pareja E, Castell JV, et al. Exploring mass spectrometry suitability to examine human liver graft metabolomic profiles. Transplant Proc. 2010;42:2953–8.
Cortes M, Pareja E, García-Cañaveras JC, et al. Metabolomics discloses donor liver biomarkers associated with early allograft dysfunction. J Hepatol. 2014;61:564–74.
Dang L, White DW, Gross S, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature. 2009;462:739–44.
Defamie V, Cursio R, Le Brigand K, et al. Gene expression profiling of human liver transplants identifies an early transcriptional signature associated with initial poor graft function. Am J Transplant. 2008;8:1221–36.
Dettmer K, Aronov AP, Hammock BD. Mass spectrometry-based metabolomics. Mass Spectrom Rev. 2007;26:51–78.
Duarte IF, Stanley EG, Holmes E, et al. Metabolic assessment of human liver transplants from biopsy samples at the donor and recipient stages using high-resolution magic angle spinning 1H NMR spectroscopy. Anal Chem. 2005;77:5570–8.
Dunn WB. Current trends and future requirements for the mass spectrometric investigation of microbial mammalian and plant metabolomes. Phys Biol. 2008;5:011001.
Dunn WB, Bailey NJ, Johnson HE. Measuring the metabolome: current analytical technologies. Analyst. 2005;130:606–25.
Ericzon B, Eusufzai S, Kubota K, et al. Characteristics of biliary lipid metabolism after liver transplantation. Hepatology. 1990;12:1222–8.
Fahy E, Sud M, Cotter D, et al. LIPID MAPS online tools for lipid research. Nucleic Acids Res. 2007;35:W606–12.
Fan X, Bai J, Shen P. Diagnosis of breast cancer using HPLC metabolomics fingerprints coupled with computational methods. Conf Proc IEEE Eng Med Biol Soc. 2005;6:6081–4.
Fiehn O. Metabolomics–the link between genotypes and phenotypes. Plant Mol Biol. 2002;48:155–71.
Fondevila C, Hessheimer AJ, Maathuis MH, et al. Superior preservation of DCD livers with continuous normothermic perfusion. Ann Surg. 2011;254:1000–7.

Fouassier L, Beaussier M, Schiffer E, et al. Hypoxia-induced changes in the expression of rat hepatobiliary transporter genes. Am J Physiol Gastrointest Liver Physiol. 2007;293: G25–35.

Francis H, Meng F, Gaudio E, et al. Histamine regulation of biliary proliferation. J Hepatol. 2012;56:1204–6.

García-Cañaveras JC, Donato MT, Castell JV, et al. A comprehensive untargeted metabolomic analysis of human steatotic liver tissue by RP and HILIC chromatography coupled to mass spectrometry reveals important metabolic alterations. J Proteome Res. 2011;10:4825–34.

Garcia-Cañaveras JC, Donato MT, Castell JV, et al. Targeted profiling of circulating and hepatic bile acids in human, mouse, and rat using a UPLC-MRM-MS-validated method. J Lipid Res. 2012;53:2231–41.

García-Cañaveras JC, Jiménez N, Gómez-Lechón MJ, et al. LC-MS untargeted metabolomic analysis of drug-induced hepatotoxicity in HepG2 cells. Electrophoresis. 2015;36:2294–302.

Garcia-Cañaveras JC, López S, Castell JV, et al. Extending metabolome coverage for untargeted metabolite profiling of adherent cultured hepatic cells. Anal Bioanal Chem. 2016;408: 1217–30.

Garcia-Valdecasas JC, Tabet J, Valero R, et al. Evaluation of ischemic injury during liver procurement from non-heart-beating donors. Eur Surg Res. 1999;31:447–56.

Gibelin H, Eugene M, Hebrard W, et al. A new approach to the evaluation of liver graft function by nuclear magnetic resonance spectroscopy. A comparative study between Euro-Collins and University of Wisconsin solutions. Clin Chem Lab Med. 2000;38:1133–6.

Goldsmith P, Fenton H, Morris-Stiff G, et al. Metabolomics: a useful tool for the future surgeon. J Surg Res. 2010;160:122–32.

Gomez-Lechon MJ, Lahoz A, Gombau L, et al. In vitro evaluation of potential hepatotoxicity induced by drugs. Curr Pharm Des. 2010;16:1963–77.

Han MS, Park SY, Shinzawa K, et al. Lysophosphatidylcholine as a death effector in the lipoapoptosis of hepatocytes. J Lipid Res. 2008;49:84–97.

Hauet T, Baumert H, Gibelin H, et al. Noninvasive monitoring of citrate, acetate, lactate, and renal medullary osmolyte excretion in urine as biomarkers of exposure to ischemic reperfusion injury. Cryobiology. 2000;41:280–91.

Hedaya MS, El Moghazy WM, Yasutomo Y, et al. Is biliary bile acid a good predictor for acute cellular rejection in living donor liver transplantation? Hepatobiliary Pancreat Dis Int. 2009;8:474–8.

Henderson JM. Liver transplantation and rejection: an overview. Hepatogastroenterology. 1999;46:1482–4.

Holland NT, Smith MT, Eskenazi B, et al. Biological sample collection and processing for molecular epidemiological studies. Mutat Res. 2003;543:217–34.

Holmes E, Bonner FW, Sweatman BC, et al. Nuclear magnetic resonance spectroscopy and pattern recognition analysis of the biochemical processes associated with the progression of and recovery from nephrotoxic lesions in the rat induced by mercury (II) chloride and 2-bromoethanamine. Mol Pharmacol. 1992;42:922–30.

Horai H, Arita M, Kanaya S, et al. MassBank: a public repository for sharing mass spectral data for life sciences. J Mass Spectrom. 2010;45:703–14.

Howard P, Murphy G. Bile physiology: theory and practice. Curr Opin Gastroenterol. 1990;6:657–67.

Hrydziuszko O, Silva MA, Perera MT, et al. Application of metabolomics to investigate the process of human orthotopic liver transplantation: a proof-of-principle study. Omics. 2010; 14:143–50.

Hylemon PB, Zhou H, Pandak WM, et al. Bile acids as regulatory molecules. J Lipid Res. 2009;50:1509–20.

Ibanez C, Simo C, Garcia-Canas V, et al. CE/LC-MS multiplatform for broad metabolomic analysis of dietary polyphenols effect on colon cancer cells proliferation. Electrophoresis. 2012;33:2328–36.

Idborg-Bjorkman H, Edlund PO, Kvalheim OM, et al. Screening of biomarkers in rat urine using LC/electrospray ionization-MS and two-way data analysis. Anal Chem. 2003; 75:4784–92.
Kaddurah-Daouk R, Kristal BS, Weinshilboum RM. Metabolomics: a global biochemical approach to drug response and disease. Annu Rev Pharmacol Toxicol. 2008;48:653–83.
Kakisaka K, Cazanave SC, Fingas CD, et al. Mechanisms of lysophosphatidylcholine-induced hepatocyte lipoapoptosis. Am J Physiol Gastrointest Liver Physiol. 2012;302:G77–84.
Kukan M, Haddad PS. Role of hepatocytes and bile duct cells in preservation reperfusion injury of liver grafts. Liver Transpl. 2001;7:381–400.
Lahoz A, Gombau L, Donato MT, et al. In vitro ADME medium/high-throughput screening in drug preclinical development. Mini Rev Med Chem. 2006;6:1053–62.
Lenz ME, Wilson ID. Analytical strategies in metabolomics. J Proteome Res. 2007;6:443–58.
Lenz EM, Bright J, Knight R, et al. Cyclosporin A-induced changes in endogenous metabolites in rat urine: a metabolomic investigation using high field 1HNMR spectroscopy. HPLCTOF/MS and chemometrics. J Pharm Biomed Anal. 2004;35:599–608.
Leon Z, Garcıa-Canaveras JC, Donato MT, et al. Mammalian cell metabolomics: experimental design and sample preparation. Electrophoresis. 2013;34:2762–75.
Li H, Wang L, Yan X, et al. A proton nuclear magnetic resonance metabolomics approach for biomarker discovery in nonalcoholic fatty liver disease. J Proteome Res. 2011;10:2797–806.
Lindon JC, Holmes E, Bollard ME, et al. Proton nuclear magnetic resonance analysis of hepatic bile from donors and recipients in human liver transplantation. Biomarkers. 2004;9:1–31.
López-Ibáñez J, Pazos F, Chagoyen M. MBROLE 2.0 – functional enrichment of chemical compounds. Nucleic Acids Res. 2016;44:W201–4.
Mao Y, Yu J, Chen J, et al. Diagnosis of renal allograft subclinical rejection by urine protein fingerprint analysis. Transpl Immunol. 2008;18:255–9.
Martin-Sanz P, Bosca L, Olmedilla L, et al. Presence of a nitric oxide synthase inhibitor in the graft efflux during reperfusion in human liver transplantation. Clin Transplant. 1999;13:221–30.
Martin-Sanz P, Olmedilla L, Dulin E, et al. Presence of methylated arginine derivatives in orthotopic human liver transplantation: relevance for liver function. Liver Transpl. 2003;9:40–8.
Melendez HV, Heaton ND. Understanding. “marginal” liver grafts. Transplantation. 1999;68:469–71.
Melendez HV, Ahmadi D, Parkes HG, et al. Proton nuclear magnetic resonance analysis of hepatic bile from donors and recipients in human liver transplantation. Transplantation. 2001;72:855–60.
Motoki A, Adachi N, Liu K, et al. Suppression of ischaemia-induced cytokine release by dimaprit and amelioration of liver injury in rats. Basic Clin Pharmacol Toxicol. 2008;102:394–8.
Mouly-Bandini A, Vion-Dury J, Viout P, et al. Detection of acute cardiac rejection by high resolution proton magnetic resonance spectroscopy of plasma. MAGMA. 2000;11:27–32.
Navarro-Sabate A, Peralta C, Calvo M, et al. Mediators of rat ischemic hepatic preconditioning after cold preservation identified by microarray analysis. Liver Transpl. 2006;12:1615–25.
Naz S, Vallejo M, García A, et al. Method validation strategies involved in non-targeted metabolomics. J Chromatogra A. 2014;1:1353–99.
Nicholson JK, Lindon JC. Systems biology: metabolomics. Nature. 2008;455:1054–6.
Nicholson JK, Lindon JC, Holmes E. ‘Metabolomics’: understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica. 1999;29:1181–9.
Nicholson JK, Connelly J, Lindon JC, et al. Metabolomics: a platform for studying drug toxicity and gene function. Nat Rev Drug Discov. 2002;1:153–61.
Nowak G, Ungerstedt J, Wernerman J, et al. Metabolic changes in the liver graft monitored continuously with microdialysis during liver transplantation in a pig model. Liver Transpl. 2002;8:424–32.
Odunsi K, Wollman RM, Ambrosone CB, et al. Detection of epithelial ovarian cancer using 1H-NMR-based metabolomics. Int J Cancer. 2005;113:782–8.

Ogata H, Goto S, Sato K, et al. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 1999;27:29–34.
Oliver SG, Winson MK, Kell DB, et al. Systematic functional analysis of the yeast genome. Trends Biotechnol. 1998;16:373–8.
Olthoff KM, Kulik L, Samstein B, et al. Validation of a current definition of early allograft dysfunction in liver transplant recipients and analysis of risk factors. Liver Transpl. 2010;16:943–9.
Pham-Tuan H, Kashavelis L, Daykin CA, et al. Method development in high-performance liquid chromatography for high throughput profiling and metabolomic studies of biofluid samples. J Chromatogr B Analyt Technol Biomed Life Sci. 2003;789:283–301.
Plumb RS, Stumpf CL, Gorenstein MV, et al. Metabolomics: the use of electrospray mass spectrometry coupled to reversed-phase liquid chromatography shows potential for the screening of rat urine in drug development. Rapid Commun Mass Spectrom. 2002;16:1991–6.
Psychogios N, Hau DD, Peng J, et al. The human serum metabolome. PLoS One. 2011;6:6e16957.
Puri P, Baillie RA, Wiest MM, et al. A lipidomic analysis of nonalcoholic fatty liver disease. Hepatology. 2007;46:1081–90.
Puri P, Wiest MM, Cheung O, et al. The plasma lipidomic signature of nonalcoholic steatohepatitis. Hepatology. 2009;50:1827–38.
Quintás G, Portillo N, García-Cañaveras JC, et al. Chemometric approaches to improve PLSDA model outcome for predicting human non-alcoholic fatty liver disease using UPLC-MS as a metabolic profiling tool. Metabolomics. 2012;8:86–98.
R Core Team. R Foundation for Statistical Computing V, Austria. R: a language and environment for statistical computing. 2014. ISBN 3-900051-07-0. URL http://www.R-project.org/2012
Raza A, Dikdan G, Desai KK, et al. Global gene expression profiles of ischemic preconditioning in deceased donor liver transplantation. Liver Transpl. 2010;16:588–99.
Rhee EP, Cheng S, Larson MG, et al. Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. J Clin Invest. 2011;121:1402–11.
Robertson DG. Metabolomics in toxicology: a review. Toxicol Sci. 2005;85:809–22.
Sabatine MS, Liu E, Morrow DA, et al. Metabolomic identification of novel biomarkers of myocardial ischemia. Circulation. 2005;112:3868–75.
Sakka SG. Assessing liver function. Curr Opin Crit Care. 2007;13:207–14.
Salvalaggio P, Afonso RC, Felga G, et al. A proposal to grade the severity of early allograft dysfunction after liver transplantation. Einstein. 2013;11:23–31.
Sanins SM, Nicholson JK, Elcombe C, et al. Hepatotoxin-induced hypertaurinuria: a proton NMR study. Arch Toxicol. 1990;64:407–11.
Saude EJ, Lacy P, Musat-Marcu S, et al. NMR analysis of neutrophil activation in sputum samples from patients with cystic fibrosis. Magn Reson Med. 2004;52:807–14.
Saxena V, Gupta A, Nagana Gowda GA, et al. 1H-NMR spectroscopy for the prediction of therapeutic outcome in patients with fulminant hepatic failure. NMR Biomed. 2006; 19:521–6.
Serkova NJ, Fuller TF, Klawitter J, et al. 1H-NMR based metabolic signatures of mild and severe ischemia/reperfusion injury in rat kidney transplants. Kidney Int. 2005;67:1142–51.
Serracino-Inglott F, Habib NA, Mathie RT. Hepatic ischemia reperfusion injury. Am J Surg. 2001;181:160–6.
Silva MA, Richards DA, Bramhall SR, et al. A study if the metabolites of ischemia-reperfusion injury and selected amino acids in the liver using microdialysis during transplantation. Transplantation. 2007;79:828–35.
Sinclair MC, Lemmi CA, Moore TC. Elevation in urinary excretion of histamine following renal allografting in rats. J Surg Res. 1974;17:43–4.
Singh HK, Yachha SK, Saxena R, et al. A new dimension of 1H-NMR spectroscopy in assessment of liver graft dysfunction. NMR Biomed. 2003;16:185–8.
Smith CA, O'Maille G, Want EJ, et al. METLIN: a metabolite mass spectral database. Ther Drug Monit. 2005;27:747–51.

Soga T, Baran R, Suematsu M, et al. Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. J Biol Chem. 2006;281:16768–76.
Sommer T, Larsen JF. Intraperitoneal and intraluminal microdialysis in the detection of experimental regional intestinal ischaemia. Br J Surg. 2004;91:855–61.
Stenlund H, Madsen R, Vivi A, et al. Monitoring kidney-transplant patients using metabolomics and dynamic modeling. Chemom Intell Lab. 2009;98:45–50.
Strimbu K, Tavel JA. What are biomarkers? Curr Opin HIV AIDS. 2010;5:463–6.
Suarez I, Bodega G, Fernandez B. Glutamine synthetase in brain: effect of ammonia. Neurochem Int. 2002;41:123–42.
Sumner LW, Amberg A, Barrett D, et al. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics. 2007;3:211–21.
Tanaka N, Matsubara T, Krausz KW, et al. Disruption of phospholipid and bile acid homeostasis in mice with nonalcoholic steatohepatitis. Hepatology. 2012;56:118–29.
Thompson JA, Markey SP. Quantitative metabolic profiling of urinary organic acids by gas chromatography–mass spectrometry: comparison of isolation methods. Anal Chem. 1975;47:1313–21.
Tietge UJ, Bahr MJ, Manns MP, et al. Plasma amino acids in cirrhosis and after liver transplantation: influence of liver function, hepatic hemodynamics and circulating hormones. Clin Transplant. 2002;16:9–17.
Tripathi P, Bala L, Saxena R, et al. 1H NMR spectroscopic study of blood serum for the assessment of liver function in liver transplant patients. J Gastrointestin Liver Dis. 2009;18:329–36.
Trushina E, Dutta T, Persson XM, et al. Identification of altered metabolic pathways in plasma and CSF in mild cognitive impairment and Alzheimer's disease using metabolomics. PLoS One. 2013;8:e63644.
Vilca Melendez H, Gilani S, Cochrane B, et al. A validated technique for the analysis of biliary bile acid secretion in donor livers prior to transplantation. Transpl Int. 1998;11:216–22.
Vilca Melendez H, Rela M, Murphy G, et al. Assessment of graft function before liver transplantation: quest for the lost ark? Transplantation. 2000;70:560–5.
Vilca Melendez H, Rela M, Setchell KD, et al. Bile acids analysis: a tool to assess graft function in human liver transplantation. Transpl Int. 2004;17:286–92.
Villas-Boas SG, Mas S, Akesson M, et al. Mass spectrometry in metabolome analysis. Mass Spectrom Rev. 2005;24:613–46.
Vogel T, Brockmann JG, Coussios C, et al. The role of normothermic extracorporeal perfusion in minimizing ischemia reperfusion injury. Transplant Rev. 2012;26:156–62.
Wen H, Yoo SS, Kang J, et al. A new NMR-based metabolomics approach for the diagnosis of biliary tract cancer. J Hepatol. 2010;52:228–33.
WHO International Programme on Chemical Safety. Biomarkers and risk assessment: concepts and principles. 1993. http://www.inchem.org/documents/ehc/ehc/ehc155.htm
Wishart DS. Metabolomics: the principles and potential applications to transplantation. Am J Transplant. 2005;5:2814–20.
Wishart DS. Metabolomics: a complementary tool in renal transplantation. Contrib Nephrol. 2008;160:76–87.
Wishart DS, Knox C, Guo AC, et al. Nucleic Acids Res. 2009; 37: D603–10.
Wishart DS, Jewison T, Guo AC, et al. HMDB 3.0–The Human Metabolome Database in 2013. Nucleic Acids Res. 2013;41:D801–7.
Wu H, Xue R, Dong L, et al. Metabolomic profiling of human urine in hepatocellular carcinoma patients using gas chromatography/mass spectrometry. Anal Chim Acta. 2009;648:98–104.
Xia J, Mandal R, Sinelnikov IV, et al. MetaboAnalyst 2.0 – a comprehensive server for metabolomic data analysis. Nucleic Acids Res. 2012;40:W127–33.
Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0 – making metabolomics more meaningful. Nucleic Acids Res. 2015;43:W251–7.

Xu J, Casas-Ferreira AM, Ma Y, et al. Lipidomics comparing DCD and DBD liver allografts uncovers lysophospholipids elevated in recipients undergoing early allograft dysfunction. Sci Rep. 2015;5:17737.

Yang J, Xu G, Zheng Y, et al. Diagnosis of liver cancer using HPLC-based metabolomics avoiding false-positive result from hepatitis and hepatocirrhosis diseases. J Chromatogr B Anal Technol Biomed Life Sci. 2004;813:59–65.

Liver Biomarkers and Their Applications to Nutritional Interventions in Animal Studies

6

Cynthia Aparecida de Castro, Manoela Maciel dos Santos Dias, Karina Ana da Silva, Sandra Aparecida dos Reis, Lisiane Lopes da Conceição, Letícia De Nadai Marcon, Luis Fernando de Sousa Moraes, and Maria do Carmo Gouveia Peluzio

Contents

Abstract

The liver plays many important functions in the metabolic processes involving the dietary aspect in health and disease. Regular intake of bioactive compounds is associated with beneficial effects on chronic diseases, including liver diseases. The aim of this chapter is to provide a comprehensive overview of the effect of nutritional interventions on liver biomarkers in animal models, with emphasis on the participation of functional foods and intestinal microbiota. An update of some

C.A. de Castro (✉) • K.A. da Silva
Department of Physiological Sciences, Federal University of São Carlos (UFSCAR), São Carlos, São Paulo, Brazil
e-mail: cynthiaefi2004@yahoo.com.br; ccastroefi@gmail.com; karinana78@yahoo.com.br

M.M. dos Santos Dias • S.A. dos Reis • L.L. da Conceição • L. De Nadai Marcon • L.F. de Sousa Moraes • M. do Carmo Gouveia Peluzio
Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil
e-mail: manoelamaciel810@gmail.com; sandraadosreis@hotmail.com; lisianelopes@yahoo.com.br; leticiadenadai@gmail.com; moraesnando@yahoo.com.br; mpeluzio@ufv.br

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_1

cytohistological and molecular biology techniques used to detect biochemical, inflammatory biomarkers are also presented.

Keywords

Liver • Biomarkers • Animal studies • Hepatic disease • Nutritional intervention • Bioactive compounds

List of Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CCl_4	Carbon tetrachloride
CRP	C-reactive protein
DMN	Dimethylnitrosamine
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenol
ECM	Extracellular matrix
ESM	Eggshell membrane
EVs	Extracellular vesicles
FFA	Free fat acids
FRU	High-fructose diet
GC	Chitin-glucan fiber
GGT	γ-Glutamil transferase
HCC	Cirrhosis and hepatocellular Carcinoma
HFD	High-fat diet
HFS	High-fat sucrose
HSCs	Hepatic stellate cells
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon gamma
IL-10	Interleukin-10
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
ITRAQ	Isobaric tags for relative and absolute quantitation
LC ESI-MS/MS	Sensitive liquid chromatography-electrospray ionization-tandem mass spectrometry
MCP-1	Monocyte chemotactic protein-1
MiRNA	Micro-RNA
MRNA	Messenger RNA
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NF- kB	Nuclear transcription factor-kB
OS	Oxidative stress
PI	Propidium iodide
qRT – PCR	Quantitative real-time polymerase chain reaction

RNA	Ribonucleic acid
RNA	Ribonucleic acid
ROS	Reactive oxygen species
TGF- β1	Transforming growth factor-β1
THC	Tetrahidrocurcumin
TNF-α	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

Key Facts of Nutrition Intervention

- Nutrition intervention using bioactive compounds, prebiotics, and probiotics in adequate amounts can exert health benefits.
- Polyphenols and probiotics can have effects, i.e., antioxidant, anti-inflammatory, and other effects in the metabolism, which contribute to the host health promotion.
- Polyphenols has antioxidant activity, protecting cells and tissues from damage caused by reactive oxygen species.
- One of the main mechanisms of action of foods compounds with anti-inflammatory function is the reduction of the expression of pro-inflammatory cytokines.
- Probiotics have a beneficial effect on the balance of the intestinal microbiota and improving the intestinal barrier function, decrease endotoxemia and inflammation, besides increasing the antioxidant defense, and stimulate the immune system.
- Prebiotic may act by lowering blood lipids, reducing triglycerides in the liver, improving glycemic control, and stimulating beneficial gut bacteria.
- The effect of nutritional interventions in vivo can be evaluated by cytohistological analysis, nutrigenomic approach, and micro-RNA profile.

Definitions of Words and Terms

Biochemical markers	Any hormone, enzyme, antibody, or other substances detected in urine, blood, or other body fluids or tissues, which may serve as a sign of a disease or other abnormality.
Cytohistology	The integrated study of cells and tissues.
Cytokines	Large group of small proteins (∼5–20 kDa), peptides, or glycoproteins secreted by specific cells of immune system, which can affect the behavior of other cells.
Endotoxemia	High endotoxin levels in the blood derived from gram-negative bacteria, which may cause inflammation.

Functional foods	Foods consumed as part of a usual diet, which can produce great physiological effects, useful in maintaining good physical and mental health, and may help to reduce the risk of chronic diseases.
Hepatobiliary	Pertaining to or emanating from the liver, bile ducts, and gallbladder.
Immunohistochemistry	Combines anatomical, immunological, and biochemical techniques to identify discrete tissue components by the interaction of target antigens with specific antibodies tagged with a visible label.
Inflammation	Is a protective response that involves immune cells, blood vessels, and molecular mediators to eliminate the initial cause of cell injury, to clear out necrotic cells and tissues damaged from the original insult, and to initiate tissue repair.
Nutrigenomics	Is the study of the effects of foods and food components on gene expression. It focuses on identifying and understanding molecular-level interaction between nutrients and other dietary bioactive compounds with the genome.
Oxidative stress	Is essentially an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants.
Prebiotics	Are typically nondigestible fiber compounds that go undigested through the upper part of the gastrointestinal tract and stimulate the growth or activity of beneficial bacteria in the large bowel by acting as substrate for them.
Probiotics	Are live microorganisms which, when administered in appropriate amounts, are able to improve the balance of intestinal microbiota, providing beneficial effects to the host health.

Introduction

Diet exerts a direct impact on health and the incidence of many chronic diseases (Elliott and Ong 2002). Nutrient deficiencies, macronutrient imbalance, or toxic concentrations of certain food compounds can adversely affect human health, while other bioactive food compounds can modulate the development of chronic illnesses, like chronic liver diseases (Chalabi et al. 2008) (Table 1).

The main chronic liver diseases include alcoholic or autoimmune viral hepatitis, nonalcoholic fatty liver disease (NAFLD), liver cirrhosis, and hepatocellular

Table 1 Experimental studies concerning the role of dietary natural compounds in liver inflammation. Interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-alpha), monocyte chemotactic protein-1(MCP-1), NF-kB (factor nuclear kappa B), interleukin-1 beta (IL-1 beta)

Dietary natural compounds	Approach/sample size	Cytokine/chemokine	References
Capsaicin	Male C57BL/6 mice ($n = 12$, 8 weeks)	↓ IL-6 and TNF-α, MCP-1 in the liver	Kang et al. (2010)
Raspberry ketone	Sprague-Dawley rats, 1:1 male to female ($n = 8$, 8 weeks)	↓ TNF-α; in serum	Wang, Meng and Zhang (2012)
Lycopene	Male Sprague-Dawley ($n = 10$, 6 weeks)	↓ TNF-α in serum	Bahcecioglu et al. (2010)
Green tea extract	Male Wistar rats ($n = 63$;16 week old)	↓ NF-kB; TNF-α; MCP-1 in liver	Park et al. (2012)
Probiotics	Zucker-Lepr$^{fa/fa}$ ($n = 40$, 30 days)	↓ TNF-α and IL-6 in serum	Plaza-Diaz et al. (2014)
Probiotics	Male Fischer ($n = 6$, 16 weeks)	↓ TNF-α in liver	Endo et al. (2013)
Yam peel extract	Male Wistar rats ($n = 8$, 8 weeks)	↓ TNF-α/NF-kB in serum	Yeh et al. (2013)
Curcumin	Male New Zealand rabbits ($n = 7$, 30 days)	↓ TNF-α in liver	Ramirez-Tortosa et al. (2009)
Resveratrol	Male Long-Evans rats ($n = 5$, 7 days)	↓ NF-kB and IL-1 β in liver	Chang et al. (2012)

carcinoma. Among such diseases, NAFLD presents one of the highest incidences worldwide (Abenavoli and Peta 2014).

Several methods are used to identify these liver disorders, such as biochemical and histological techniques. Further, more recently, transcriptomics, proteomics, and metabolomics have been used to assess, respectively, messenger ribonucleic acid (mRNA) changes, protein modifications, and the nutrient/metabolite transport of specific biomarkers. Such biomarkers of liver damage must be specific to the liver and bear strong correlation with liver histomorphological changes. Besides, they can be used in screening trials by high-throughput methods and in preclinical assay with different experimental models (Ozer et al. 2008).

Several dietary compounds isolated from fruits and vegetables possess anti-inflammatory and antioxidant properties that promote good health. Some studies show that the consumption of foods in the presence of probiotics/prebiotics, besides bioactive compounds, such as quercetin, rutin, catechin, methylxanthine, resveratrol, polyphenols, and flavonoids, can prevent oxidative stress and decrease morbidity and mortality in chronic liver diseases.

In this chapter, the biochemical, histological, immunological, and molecular mechanisms, along with nutritional interventions, will be discussed to enhance the prognostic outcomes for liver disease in animal models.

Biochemical Markers of Liver Disorders

Biochemical markers have been utilized as reliable predictors in the estimation of the nutritional status of a patient. Among the methods available, the serum or plasma levels of the liver enzymes involved in key metabolic pathways are routinely used (Ramaiah 2007). As quantifying the liver enzymes is noninvasive, easy, and inexpensive to perform, other more expensive markers are used only when the liver enzymes themselves are altered.

It is important to observe that any increase in the serum liver enzymes depends upon its (a) activity in the liver tissue, (b) cellular localization, (c) plasmatic clearance rate, and especially (d) the type, severity, and time of occurrence of liver damage (Ramaiah 2007).

The enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and γ-glutamyl transferase (GGT) are the most widely used biochemical markers in nutritional intervention studies for the evaluation of liver damage (Ozer et al. 2008).

The ALT and AST enzymes are involved in the metabolism of both amino acids and gluconeogenesis. They are responsible for the transamination process, which catalyzes the transfer of the α-amino groups to the α-ketoglutarate (Ozer et al. 2008).

The ALT enzyme is found in higher quantities in the cell membrane of the hepatocytes and is considered the gold standard biochemical marker for liver damage (Oliveira et al. 2015). However, its serum concentrations do not always correlate with the histomorphological damages present in liver tissue (Ozer et al. 2008). In relation to nonalcoholic hepatic steatosis, for example, the ALT serum concentration has only 40% diagnosis sensitivity. Therefore, additional markers need to be quantified in order to better identify the damage present (Wieckowska et al. 2007).

The AST enzyme occurred in high concentrations within the mitochondria of several tissues, such as the skeletal muscle, heart, liver, kidney, and pancreas. In contrast, this enzyme does not constitute a specific marker for liver damage (Ozer et al. 2008). Besides, the AST half-life is about 40–60 h, whereas the half-life of the ALT enzyme is only 12 h (Ramaiah 2007). Therefore, utilization of the AST/ALT ratio is recommended. A ratio value greater than 1 is usually indicative of hepatic damage (Ozer et al. 2008).

The specificity of the AST enzyme as a biochemical marker of liver damage can be increased, as this enzyme possesses two isoenzymes, differently distributed in the body tissues. The GPT1 isoenzyme (AST1) is mainly found in the kidney, liver, fat, and heart. The GPT2 (AST2) is found in greater amounts in the muscle, adipose tissue, brain, and kidney. Both isoenzymes have the same distribution in the tissues of mice and rats, the major animal models used in experimental studies for nutritional intervention (Ozer et al. 2008).

The ALP and GGT enzymes are used as the complementary markers of ALT (Ozer et al. 2008). However, ALP is not a specific marker of liver damage, because of its high concentrations in several tissues, including those of the heart, liver, skeletal muscle, kidney, and pancreas (Brun-Heath et al. 2011). The ALP associated with the cell membrane is responsible for the hydrolysis of the monophosphate

compounds under alkaline pH conditions. ALP is the most commonly used marker for hepatobiliary damage and in the diagnosis of cholestasis, a pathophysiological decrease or total interruption of bile flow to the duodenum (Ozer et al. 2008).

The GGT plays multiple roles in the body, as it occurs in various body tissues, and is therefore not a specific marker of liver damage. However, GGT is a good marker of hepatobiliary injury, especially with respect to cholestasis (Ozer et al. 2008).

In nutritional intervention studies with NAFLD, all these biochemical markers are widely used. In this condition, the serum concentrations of ALT and AST are increased, which is the first signal in NAFLD diagnosis. This occurs due to a disruption in the hepatocytes, caused by oxidative stress and lipid peroxidation during the development of the disease (Bernal et al. 2013).

As oxidative stress is closely associated with the progression of NAFLD, nutritional intervention studies in this field usually focus on the antioxidant activity of the compounds and nutrients evaluated and hence on its influence over those biochemical markers (Bernal et al. 2013). Thus, the experimental studies on NAFLD assessing the effect of the consumption of certain phenolic compounds (flavonoids and phenolic acids) have shown a drop in the serum ALT, AST, ALP, and GGT. In fact, positive results have been attributed to the antioxidant activity of those compounds (Fig. 1). The phenolic compounds are capable of stimulating the activity of the enzymes of the antioxidant system, viz., catalase, superoxide dismutase, and glutathione peroxidase. Consequently, there is a reduction in the lipid peroxidation markers (thiobarbituric acid-reactive substances, isoprostanes, and lipid hydroperoxides) as well as protein oxidation (carbonyl protein). Thereby, liver damage is also decreased (Novello et al. 2015; Oliveira et al. 2015; Wang et al. 2009b).

Rats fed on a diet containing 60% fructose and treated with genistein isoflavone (1 mg/kg/day) for 60 days showed a drop in the serum concentrations of AST, ALT, and GGT, thus improving liver function. Besides an increase in the enzymatic and nonenzymatic antioxidant defenses, a reduction in the oxidative stress markers was also observed in the group treated with genistein when compared with the control (Salih et al. 2009).

Currently, it is known that qualitative and quantitative changes in the composition of the intestinal microbiota have been associated with the induction and progression of liver diseases, particularly NAFLD (Cesaro et al. 2011). Therefore, probiotics have been increasingly studied for liver disease prevention. By definition, probiotics are "live microorganisms, which when consumed in adequate amounts confer health benefits on the host" (FAO/WHO 2001).

Nutritional intervention studies using microorganisms or probiotic foods have observed their ability to reduce serum ALT, AST, ALP, GGT (Fig. 1), and, consequently, the severity of liver damage (Bhathena et al. 2013; Nardone et al. 2010). These positive effects are related to many factors: (a) the decrease in both endotoxemia (high serum lipopolysaccharide concentration) and inflammation, (b) the increase in antioxidant defenses, (c) the improvement of the intestinal barrier function, and (d) the changes in some routes related to lipid metabolism. All these effects result from a modulation in the quantitative and qualitative changes in composition of the host intestinal microbiota (Ferolla et al. 2014).

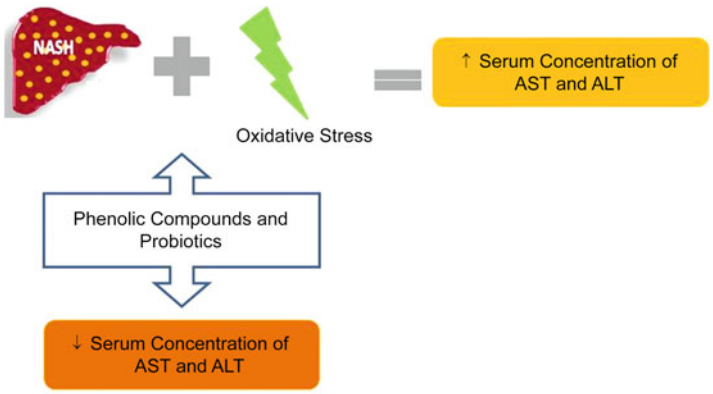

Fig. 1 Biochemical markers and nutritional intervention in NAFLD. The intense oxidative stress in NAFLD disease causes disruption of the hepatocytes. Therefore, there is a leakage of the enzymes AST and ALT into the bloodstream. However, the nutritional intervention with food rich in phenolic compounds or probiotics regulates the oxidative balance. Thus, the hepatic damage is reduced, leading to the reduction in serum concentration of AST and ALT. *AST* aspartato aminotransferase, *ALT* alanina aminotransferase, *NAFLD* nonalcoholic fatty liver disease

A high-fat diet (HFD)-induced NAFLD in hamsters treated with microencapsulated *Lactobacillus fermentum* (11.51 log CFU/mL) for 12 weeks showed no changes in the serum AST and ALP. Nevertheless, the ALT and GGT levels were reduced. Furthermore, the probiotic was also able to decrease the lipid deposition in the liver and the expression of the key enzymes involved in the hepatic lipid synthesis, such as HMG-CoA reductase (Bhathena et al. 2013).

In conclusion, ALT, AST, ALP, and GGT have sufficient predictive ability for the diagnosis of noninvasive liver disorders, especially when it is not possible to perform a biopsy. However, in some cases, the use of additional biomarkers is recommended due to the limitations of such markers (Chung et al. 2014).

Inflammatory Markers of Liver Disease

The disruption of the metabolic pathways between the adipose tissue, muscle, and liver is closely associated with the pathogenesis of NAFLD (Liu et al. 2007). Regarding these changes, the liver initiates a chronic inflammatory process and tissue remodeling. The main characteristic of these processes is the accumulation of the extracellular matrix proteins, triggered by an imbalance between the synthesis and degradation of its components (collagen). This imbalance results in an extended alteration of the hepatic parenchyma, with fibrosis formation, which, in turn, intensifies the whole inflammatory process (Mallat and Lotersztajn 2013). Thus, higher quantities of fatty acid are converted into triglycerides, which are responsible for the lipid droplet formation in the cytoplasm of the hepatocytes. This process enhances the oxidative stress and the activation of the inflammatory pathways

(Ong et al. 2005), with greater expression of the proinflammatory cytokines, observed in both acute and chronic liver diseases (Tilg et al. 2006).

Non-pharmacological therapeutic strategies have been speculated upon as a possible intervention for liver disease treatment. Recently, the search for natural foods has increased worldwide. The so-called functional foods can both prevent and control such diseases. Many dietary compounds isolated from fruits and vegetables have anti-inflammatory and antioxidant properties that contribute to the promotion of good health. These compounds could play a regulatory role in liver disease mechanisms (Pan et al. 2014). The major food groups, such as polyphenols, carotenoids, and probiotics, will be further discussed.

The administration of polyphenol-rich foods has been observed to decrease the risk of chronic diseases (Manach et al. 2004), such as cardiovascular diseases, diabetes, and obesity. Teas, soybean, grape, red fruits, and also apple peel are rich in polyphenols (Tsao 2010), which implies the presence of anti-inflammatory and lipid-lowering properties (Rahman et al. 2006).

Dietary supplementation with apple flavonoids (25 mg/kg/day) and fish oil (1 g/kg/day) or both significantly improved the lipid profile and inflammatory biomarkers in the HFD-fed rats. Serum concentrations of interleukin-6 (IL-6) cytokine, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and C-reactive protein (CRP) were reduced, while that of the anti-inflammatory cytokine interleukin-10 (IL-10) was increased (Sekhon-Loodu et al. 2014).

Genistein, an isoflavone abundantly present in soybeans, exhibited high antioxidant and anti-inflammatory effects when the liver injuries were assessed in rats (Kuzu et al. 2007). It has been suggested that genistein (5 mg/kg/day) significantly suppresses the production of the proinflammatory cytokines, including TNF-α and interleukin-1 beta (IL-1β), and inhibits early apoptosis, thereby preventing cell and tissue damage (Ganai et al. 2015).

Resveratrol, a dietary polyphenol present in grapes, peanuts, and red wine, can prevent hepatocarcinogenesis by the suppression of inflammation and oxidative stress in rats. The treatment with resveratrol (50 mg/kg) reduces the hepatic expression of TNF-α, IL-1β, and IL-6 (Mbimba et al. 2012).

Another important polyphenol is hesperidin, present in citrus fruits like orange and tangerine. Recent studies have shown that hesperidin (2 g/100 g diet) protects against hepatotoxicity and improves hepatic steatosis and hypertriglyceridemia by inhibiting the activity of the lipogenic enzymes and activating fatty acid oxidation in the liver. These beneficial effects could be related to the increased levels of adiponectin and IL-10 and the decreased levels of the pro-inflammatory markers, such as IL-6, monocyte chemotactic protein-1 (MCP-1), INF-γ, and TNF-α in the plasma or liver (Park et al. 2013). It is noteworthy that adiponectin (also called ACRP30 and GBP28) is a protein exclusively secreted by the adipose tissue and has anti-inflammatory and hepatoprotective effects (Berg et al. 2002; Yokota et al. 2000).

Carotenoids, the natural compounds mainly present in yellow fruits and vegetables, have significant effects on human health due to their antioxidative action, thus protecting the cells and tissues from damage caused by the reactive oxygen species (ROS) (Rao and Rao 2007).

Studies have shown that tomato extract and the amount of available lycopene are effective in protecting the liver from HFD-induced inflammation. They can also reduce the pro-inflammatory cytokine expression (Wang et al. 2010). Eight-week-old male rats fed on an HFD expressed a higher inflammatory focus number when compared with the controls receiving the chow diet. The inflammatory foci were reduced by adding tomato extract (250 mg TE/kg BW per day) to the HFD. However, the same effect was not observed in the group administered lycopene alone (15 mg LY/kg BW per day). Besides, the tomato extract, but not the lycopene, was also able to reduce the TNF-α, IL-1β, and IL-12 levels (Wang et al. 2010).

Different peach-derived products (peel, pulp, and syrup) also have provided beneficial effects in liver inflammation caused by carbon tetrachloride (CCl_4). The oral administration for 30 days conferred significant protection by preventing lipid and protein oxidative damage, as well as inhibiting the inflammatory mediators such as TNF-α, IL-1β, and nuclear transcription factor-kB (NF-kB) (Gasparotto et al. 2014).

Probiotics are able to restore intestinal microbiota. They stimulate immunity and protect the intestinal barrier (Nardone et al. 2010). Prebiotics are nondigestible food components, such as inulin and fructooligosaccharide, which have favorable effects, by modulating the intestinal microbiota composition, the lipid metabolism, and the gut barrier function (Miura and Ohnishi 2014).

Probiotics can modulate the immune system by stimulating the production of anti-inflammatory cytokines, such as IL-10 and TGF-β, and suppressing the pro-inflammatory cytokines, such as TNF-α, thus reducing oxidative and inflammatory damage in both the bowel and liver (Lo et al. 2014). Prebiotics may act by lowering the blood lipid levels, reducing the triglycerides in the liver, and improving glycemic control (Parnell et al. 2012).

Thus, both probiotics and prebiotics can cause changes in the intestinal microbiota, which in turn increase the concentration of short-chain fatty acids, whose anti-inflammatory properties can contribute toward attenuation of liver damage. Moreover, some intestinal bacteria are a source of Toll-like receptors, and their modulation can increase the amount of these receptors in the liver (Fig. 2). Such receptors stimulate the liver cells to produce pro-inflammatory cytokines. Thus, the normalization of the intestinal microbiota using probiotics and prebiotics opens up as a new promising option in liver disease treatment (Miura and Ohnishi 2001). Animal studies with nonalcoholic hepatitis showed that treatment with probiotics can effectively reduce liver damage by increasing the adiponectin and improving the insulin sensibility (Savcheniuk et al. 2014; Everard et al. 2014). Nevertheless, the exact mechanism is yet to be fully elucidated.

The administration of *Saccharomyces boulardii* (120 mg/day), a probiotic species, can alter the intestinal microbiota and reduce hepatic steatosis, besides systemic and liver inflammation, in diabetic mice. These changes are related to a reduction in the expression of MCP-1, hepatic IL-1, and plasma IL-6, IL-1β, and TNF-α (Everard et al. 2014). Furthermore, hepatic damage was reduced when a probiotic preparation, consisting of *Streptococcus thermophilus* and various lactobacilli and bifidobacterial species, was administered (Esposito et al. 2009).

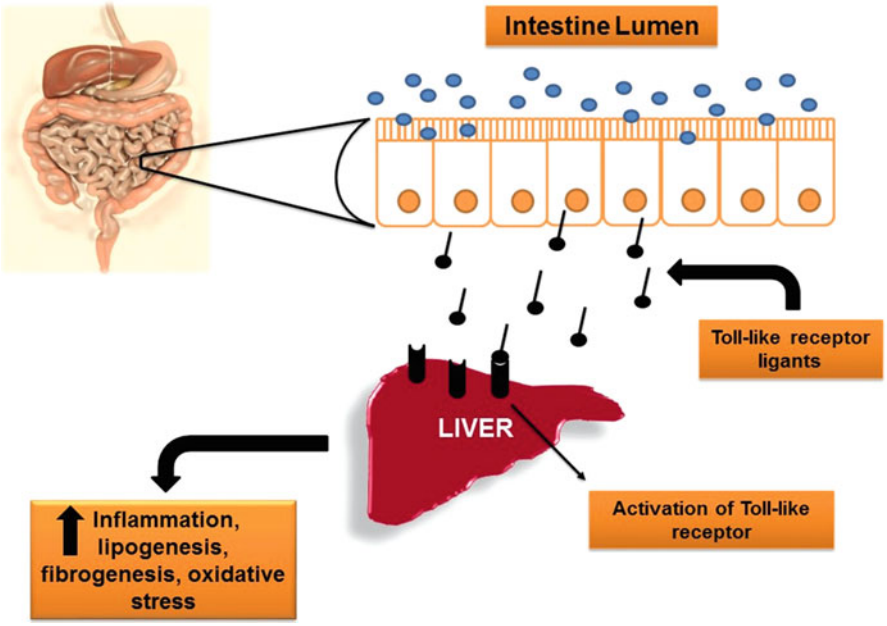

Fig. 2 Mechanisms involved in the relationship between intestinal microbiota and liver. The intestinal microbiota plays an important role in the gut-liver axis. The increase of pathogenic bacteria in intestinal lumen can disrupt the intestinal barrier function. Therefore, the increase of intestinal permeability can lead to a release of toll-like receptor (*TLR*) ligands, which can bind to toll-like receptors (*TLRs*) in the liver, inducing hepatic inflammation, lipogenesis, fibrogenesis, and oxidative stress

Regarding prebiotics, a mixture of fermentable dietary fiber (oligofructose) was able to reduce the plasma cytokines, such as IL-1 α, IL-1 β, IL-6, IL-10, TNF-α, MCP-1, and INF-γ in animals. This alteration in the inflammatory markers might be associated with greater intestinal permeability and integrity of the cell junctions (Cani et al. 2009). Finally, food plays a crucial role in the treatment and prevention of liver diseases by reducing the inflammatory markers and oxidative stress. Figure 3 shows the interaction between the inflammatory markers, functional foods, probiotics, and prebiotics involved in liver disease treatment. It has been found that physical inactivity and poor quality of diet (lifestyle) are factors that compromise health and can still induce fat accumulation in the liver. This fat accumulation may be due to an increased mobilization of free fatty acids, which increases the de novo synthesis of fatty acids and triglycerides in the liver. Besides, the increase in the levels of TNF-α and IL-6 and the decrease in the adiponectin level result in steatosis. Probiotics, prebiotics, and functional foods can reduce the levels of these cytokines as well as those of the adipokines, thus diminishing their deleterious effects in the liver.

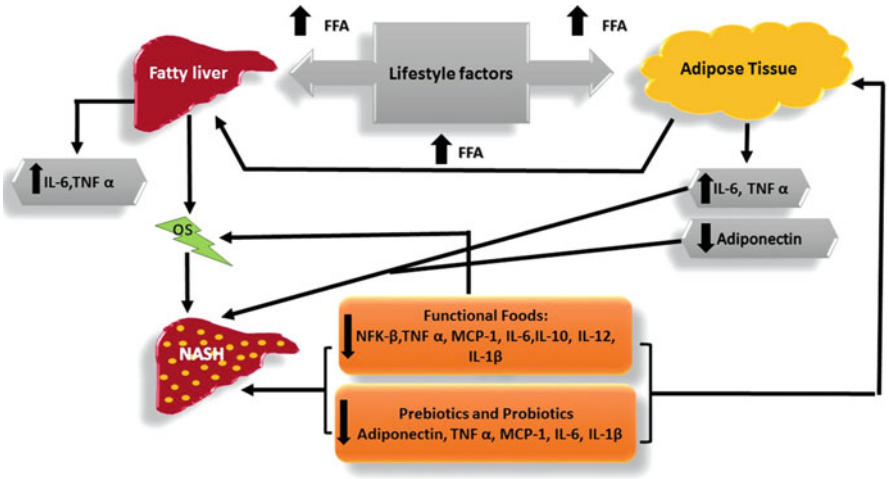

Fig. 3 Link between biomarkers, probiotic, prebiotic, and functional foods in liver disease. *FFA* free fat acids, *IFN-γ* interferon gamma, *IL-1β* interleukin-1 beta, *IL-6* interleukin – 6, *IL-10* interleukin-10, *IL-12* interleukin-12, *TNF-α* tumor necrosis factor alpha, *MCP-1* monocyte chemotactic protein-1, *NF-kB* nuclear factor-kappa B, *OS* oxidative stress

Cytohistological Analysis as a Tool in the Assessment of Liver Abnormalities

Histopathological analysis is common in animal studies and enhances the understanding of the physical and functional changes. Nutritional intervention studies regarding liver diseases can be evaluated by detecting the biomarkers of steatosis, oxidative stress, inflammation, fibrosis, cell proliferation, and apoptosis, for example. Histochemical, immunohistochemical, and immunofluorescence techniques not only contribute toward the detection of such biomarkers but also assist in the whole pathological process involved. In Figs. 4 and 5, the histological photomicrographs of the liver tissue are shown under different staining/marking techniques, prior to and post each treatment.

The search for triglyceride accumulation in the liver tissue can be performed using the technique Oil Red O, which stains the tissue lipids red. In an intervention study, mice were given HFD. The addition of chitin-glucan fiber to the diet was capable of decreasing the accumulation of hepatic lipids, which was confirmed by the lower degree of staining with Oil Red O (Fig. 4a) (Neyrinck et al. 2012).

Factors involved in the pathogenesis of liver diseases, such as the oxidative stress, have been widely studied in relation to the antioxidant components of the diet (Eslamparast et al. 2015). Some biomarkers of oxidative stress, such as 4-hydroxynonenal (a marker of lipid peroxidation), 2,4-dinitrophenol (a marker of protein oxidation), and 3-nitrotyrosine (a nitrosative stress marker), can be assessed

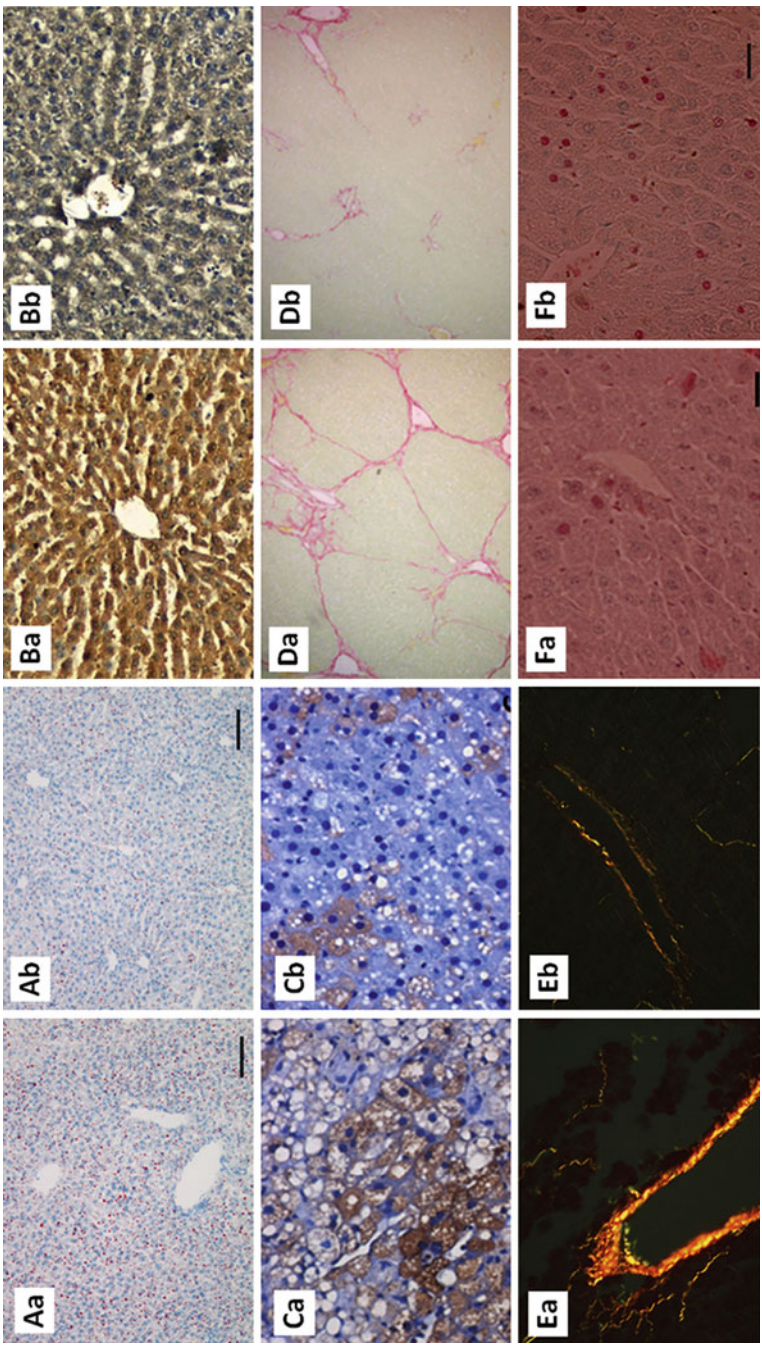

Fig. 4 Histological photomicrographs of liver tissue under different staining or marking techniques. (**A**) Histochemistry with Oil Red O. *Red dots* indicate lipids staining (a, HFD; b, HFD + supplementation CG) (Neyrinck et al. 2012) (Reprinted from Journal of Nutritional Biochemistry, v.23, Audrey M. Neyrinck, Sam Possemiers, Willy Verstraete, Fabienne De Backer, Patrice D. Cani, Nathalie M. Delzenne, Dietary modulation of clostridial cluster XIVa gut bacteria (Roseburia spp.) by chitin-glucan fiber improves host metabolic alterations induced by high-fat diet in mice, p. 51–59, Copyright (2015), with permission from

by immunohistochemistry on the liver tissue. Rats fed on a high-fructose diet and supplemented with naringenin (50 mg/kg), a flavanone present in citrus fruits, were able to decrease the immunoreactivity of these biomarkers (Fig. 4b) (Kannappan et al. 2010).

Histology can also contribute toward a better understanding of the signaling pathways of the inflammatory processes by combining molecular techniques with immunohistochemistry. For example, immunohistochemistry revealed that an intervention with 1,25(OH)2D3 (Miura and Ohnishi 2014) (active form of vitamin D) in diabetic animals was able to reduce the MCP-1, ICAM-1 (intercellular adhesion molecule-1), and transforming growth factor-β1 (TGF-β1); this implies the anti-inflammatory role played by this vitamin in the liver of such animals (Fig. 4c) (Ning et al. 2015).

Hepatic fibrosis can be evaluated by detecting collagen using the Sirius Red staining technique. Rats induced with hepatic liver fibrosis were fed with garlic extract. This technique showed that the extract was able to prevent liver fibrosis when compared with the controls not fed with the garlic extract. Besides, the immunohistochemistry of the tissue transglutaminase enzyme, a biomarker of liver fibrosis, showed almost no tissue transglutaminase labeling in the animals treated with the garlic extract, suggesting an inhibitory activity of this extract on both the

Fig. 4 (continued) Elsevier). **(B)** Immunohistochemistry for DNP. *Brown marking* indicates oxidized proteins (a, FRU; b, FRU + supplementation naringenin) (Kannappan et al. 2010) (Reprinted from European Journal of Pharmacology, v. 645, Sriramajayam Kannappan, Nallasamy Palanisamy, Carani Venkatraman Anuradha, Suppression of hepatic oxidative events and regulation of eNOS expression in the liver by naringenin in fructose-administered rats, p. 177–184, Copyright (2015), with permission from Elsevier). **(C)** Immunohistochemical analysis of labeled MCP-1 in brown marking (a, diabetes; b, diabetes with vitamin D intervention) (Ning et al. 2015). **(D)** Histochemistry with Sirius Red. Collagen is in red staining (a, CCl_4; b, CCl_4 treated with garlic extract) (D'Argenio et al. 2010) (Reprinted from Digestive and Liver Disease, v. 42, Giuseppe D'Argenio, Daniela Caterina Amoruso, Giovanna Mazzone, Paola Vitaglione, Antonietta Romano, Maria Teresa Ribecco, Maria Rosaria D'Armiento, Ernesto Mezza, Filomena Morisco, Vincenzo Fogliano, Nicola Caporaso, Garlic extract prevents CCl4-induced liver fibrosis in rats: The role of tissue transglutaminase, p. 571–577, Copyright (2015), with permission from Elsevier). **(E)** Histochemistry with Sirius Red plus polarized light. Collagen is in *yellow-reddish* tones (a, hyperhomocysteinemia; b, hyperhomocysteinemia supplemented with polyphenolic extract from red wine) (Noll et al. 2011) (Reprinted from Journal of Nutritional Biochemistry, v.22, Christophe Noll, Lamia Raaf, Chris Planque, Ludovic Benard, Lise Secardin, Emile Petit, Julien Dairou, Jean-Louis Paul, Jane-Lise Samuel, Claude Delcayre, Fernando Rodrigues-Lima, Nathalie Janel, Protection and reversal of hepatic fibrosis by red wine polyphenols in hyperhomocysteinemic mice, p. 856–864, Copyright (2015), with permission from Elsevier). **(F)** Histochemistry with green methyl-pyronine. Soft bluish-green color indicates chromatin digestion (a, CCl_4; b, CCl_4 + pretreatment with almond oil) (Jia et al. 2011) (Reprinted from Food Chemistry, v. 125, Xiao-Yan Jia, Qing-An Zhang, Zhi-Qi Zhang, Yan Wang, Jiang-Feng Yuan, Hong-Yuan Wang, Di Zhao, Hepatoprotective effects of almond oil against carbon tetrachloride induced liver injury in rats, p. 673–678, Copyright (2015), with permission from Elsevier). *HFD* high-fat diet, *GC* chitin-glucan fiber, *DNP* 2,4-dinitrophenol, *FRU* high-fructose diet, *MCP-1* monocyte chemoattractant protein-1, *CCl_4* carbon tetrachloride

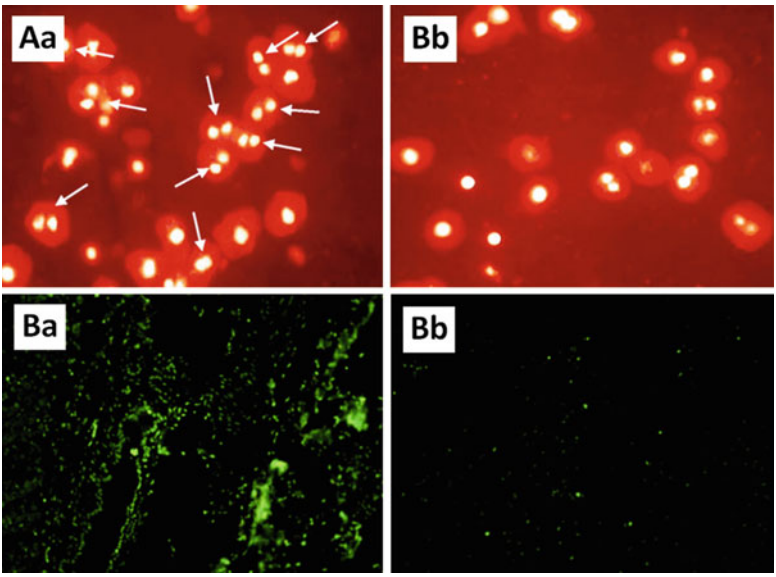

Fig. 5 Detection of apoptosis in liver cells by immunofluorescence techniques. **(A)** Immunofluorescence with PI. Fragmented cores indicate apoptosis (a, FRU; b, FRU supplemented with naringenin) (Kannappan et al. 2010) (Reprinted from European Journal of Pharmacology, v. 645, Sriramajayam Kannappan, Nallasamy Palanisamy, Carani Venkatraman Anuradha, Suppression of hepatic oxidative events and regulation of eNOS expression in the liver by naringenin in fructose-administered rats, p. 177–184, Copyright (2015), with permission from Elsevier). **(B)** TUNEL assay, cells marked in *green* indicate apoptosis (a, DMN; b, DMN treated with THC) (Weerawatanakorn et al. 2014) (Reprinted from Journal of functional foods, v. 7, Monthana Weerawatanakorn, Shu-Chen Hsieh, Mei-Ling Tsai, Ching-Shu Lai, Li-Mei Wu, Vladimir Badmaev, Chi-Tang Ho, Min-Hsiung Pan, Inhibitory effect of tetrahydrocurcumin on dimethylnitrosamine-induced liver fibrosis in rats, p. 305–313, Copyright (2015), with permission from Elsevier). *FRU* high-fructose diet, *CCl4* carbon tetrachloride, *PI* propidium iodide, *TUNEL* terminal deoxynucleotidyl transferase dUTP nick end labeling, *DMN* dimethylnitrosamine, *THC* tetrahidrocurcumin

liver enzymes and their function (Fig. 4d) (D'Argenio et al. 2010). Phenolic extract from wine was also able to reduce the collagen deposition in methionine synthase-deficient mice, the enzyme responsible for the remethylation of homocysteine to methionine, when subjected to a methionine-rich diet (Fig. 4e) (Noll et al. 2011). The liver test was performed by Sirius Red staining.

Apoptosis assays enable the assessment of the loss of cells in inflammation and hepatic fibrosis. Some staining techniques used to detect this change include methyl green-pyronin, propidium iodide, and the TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labeling). The methyl green-pyronin stains the cytoplasm of the cells red and the deoxyribonucleic acid (DNA) bluish green. Rats with liver injury fed with almond oil, rich in oleic and linoleic fatty acids, exhibited a protective effect against liver cell damage, besides showing improved histological findings (Fig. 4f) (Jia et al. 2011).

Furthermore, the untreated animals showed severe hepatic necrosis, core contraction, cytoplasmic debris, cell swelling, chromatin digestion, and organelle disruption. When high-fructose-fed rats were treated with naringenin, it was able to reduce the apoptotic nuclei formation from the hepatocytes, marked with propidium iodide (Fig.4a) (Kannappan et al. 2010).

TUNEL assay detected DNA fragmentation by labeling the nucleic acids. The hepatoprotective effect of tetrahydrocurcumin, a metabolite of curcumin, was observed in rats with fibrosis. Immunofluorescence labeling, detected by TUNEL, was reduced in the liver cells treated with tetrahydrocurcumin, suggesting the suppression of apoptosis by this compound (Fig. 4b) (Weerawatanakorn et al. 2014).

In conclusion, cytohistological analysis is also able to detect visible liver changes. Besides, they are useful tools to assess the in vivo effects of the bioactive compounds in foods.

Nutrigenomic Approaches in the Search for Biomarkers of Liver Diseases

Nutrigenomics is a high-throughput genomic tool that enables the study of the influence of nutrition on the expression of a given genome. This approach which includes three "omic" techniques, transcriptomics, proteomics, and metabolomics, facilitates the evaluation of the influence of diet on gene transcription, protein expression, and metabolism (Afman and Muller 2006; Muller and Kersten 2003; Elliott and Ong 2002). The development of nutrigenomics has provided a better understanding regarding (i) the biochemical, molecular, and cellular mechanisms that undergird the beneficial or adverse effects of certain bioactive food components; (ii) the identity of the genes involved in the stage prior to the onset of the disease and, therefore, the possible molecular biomarkers; and (iii) the effect of bioactive food constituents on crucial molecular pathways (Davis and Milner 2004; Muller and Kersten 2003). Therefore, nutrigenomics can lead to the designing of effective dietary intervention strategies to recover normal homeostasis and prevent diet-related diseases (DeBusk 2009).

Genotypes that alter disease susceptibility or resistance induced by nutritional stimuli play an important role in NAFLD (Bouchard 2008). Metabolic and hormonal responses to dietary changes involve the coordinated regulations of complex mechanisms occurring in several organs that maintain lipid homeostasis (Riserus 2008; Mann 2006). Disruption of any of these adaptive mechanisms, either through gene or environment interactions, can progressively lead to NAFLD (Li 2012).

The incidence of NAFLD has increased worldwide, but no specific biomarker is available yet, and invasive liver biopsy is still mandatory for the definite diagnosis of NAFLD, especially for nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis and hepatocellular carcinoma (HCC) (Nugent and Younossi 2007; Younossi et al. 2005). Therefore, there is an urgent need to identify the blood (serum or plasma) markers that are specific for the early diagnosis of HCC, prediction of carcinogenesis from liver cirrhosis, progression of liver cirrhosis, and diagnosis of NASH. Such

analysis may also aid in the elucidation of the mechanism(s) underlying the pathogenesis of hepatitis and hepatocarcinogenesis (Behne and Copur 2012).

High-throughput proteomic technology has allowed us to obtain the profile of a large number of proteins in biological samples. Moreover, this technique has shown that subjects with advanced liver fibrosis present different expression of a great number of protein in the serum. These proteins can be synthesized from several hepatic cell types. Thus, it can be drawn from multiple biochemical pathways, such as the carbohydrate and lipid metabolism, besides the antioxidant pathways of the hepatocytes. Different levels of such protein marker expression in the liver tissue biopsy, cell culture models, or animal models suggest that these serum proteins have a hepatic origin. Furthermore, one of the advantages of the proteomic techniques is the ability to differentiate posttranslational modifications among the proteins. Therefore, the increase of the biomarkers' specificity can improve the accuracy of the existing diagnostics (Cowan et al. 2010).

A study employing label-free quantitative proteomics detected significant changes in the protein expression levels of the hepatocyte-derived extracellular vesicles (EVs) after exposure to well-known liver toxins (galactosamine and *Escherichia coli*-derived lipopolysaccharide). The results supported the hypothesis that EVs are a suitable source of biomarkers for liver injury (Rodriguez-Suarez et al. 2014).

The nutrigenomic approach using a gene expression microarray was applied to screen the novel candidate genes for NAFLD and its prevention by methyl donor supplementation. Liver fat accumulation induced by a high-fat sucrose (HFS) diet was prevented by methyl donor supplementation in HFS-fed animals. A liver mRNA microarray, subsequently validated by quantitative real-time polymerase chain reaction (qRT-PCR), showed modifications in some biologically relevant genes involved in obesity development and lipid metabolism (Lepr, Srebf2, Agpat3, and Esr1) (Cordero et al. 2013).

A proteomic study performed by Wang et al. (2014) showed the protective effects of extra virgin olive oil (20% extra virgin olive oil mixed with rodent chow 5053) against chronic liver injury induced by CCl_4 in rats. This proteomic study found high expression of peroxiredoxin-1 and thiosulfate sulfurtransferase in the extra virgin olive oil-fed animals with CCl_4 treatment, demonstrating its strong antioxidative effects against CCl_4-induced oxidative stress. This research enhanced the understanding of the molecular mechanisms involved in the different effects of dietary lipids against chronic liver injury.

Proteomics and gene expression analysis of the mitochondria drawn from squalene-treated apoE-deficient mice identified short-chain-specific acyl-CoA dehydrogenase changes associated with fatty liver amelioration (Ramirez-Torres et al. 2012). It is noteworthy that squalene is a hydrocarbon involved in cholesterol biosynthesis and an abundant component in virgin olive oil.

A comprehensive hepatocyte-based proteomic approach was used to identify changes in the protein expression and recognize the redox-associated diabetic liver disease markers induced by high glucose concentration. High glucose concentration-modulated proteins which participate in several cellular responses, including transcription control, signal transduction, and redox regulation, as well as cytoskeleton

regulation, protein folding, and gene regulation, were identified. These findings indicated the potential markers of diabetic liver disease that are appropriate for early-stage evaluation of disease prognosis (Chen et al. 2013).

DNA microarray analysis was used to obtain nutrigenomic information. It was found that eggshell membrane (ESM) treatment markedly downregulated the expression of collagen type 1 alpha 1, smooth muscle-α-actin, integrin beta-like 1, decorin, aspirin, and lumican, the key genes involved in the activation of the hepatic stellate cells (HSCs), in rat liver. They showed that ESM treatment can suppress the HSC activation and consequent liver fibrosis in rats (Jia et al. 2013).

Isobaric tags for relative and absolute quantitation (iTRAQ) combined with sensitive liquid chromatography-electrospray ionization-tandem mass spectrometry (LC ESI-MS/MS) analyses were used to investigate the protective effect and the possible anti-fibrotic mechanism of the combination therapy with taurine (100 mg/kg), epigallocatechin gallate (15 mg/kg), and genistein (10 mg/kg) on CCl_4-induced liver fibrosis rats. A large number of differentially expressed proteins were identified. Some proteins involved in the glycolysis pathway, antioxidant defense system, coagulation cascade pathway, and inflammation were validated, which may be useful targets for the treatment of liver fibrosis in the future (Cao et al. 2015).

In conclusion, nutritional intervention can play a significant role in liver disease treatment. Moreover, the nutrigenomic approach can contribute not only to the discovery of clinically useful biomarkers but also in clarifying the molecular mechanisms of the disease pathogenesis by using body fluids, such as serum, tissue samples, and cultured cells. Thus, the early diagnosis of the stage of liver disease can also contribute to more effective therapeutic interventions and an improved prognosis.

Micro-RNA as a Tool in the Diagnosis of Liver Abnormalities

Micro-RNA (miRNA) belongs to the noncoding ribonucleic acid (RNA) class. When mature, they present as single-stranded RNA molecules having approximately 21 or 22 nucleotides (Boyd 2008). Most of the miRNA are intracellular and, therefore, critical mediators in response to cellular stress, disease, and environmental stimuli. Thus, the dysregulations of the miRNA are involved in many cellular processes such as development, differentiation, and cell signaling, as well as the pathological, physiological, and metabolic processes (Siddeek et al. 2014; Yokoi and Nakajima 2013).

The identification of blood markers may contribute to an improved knowledge of the type and/or level of liver damage. Contrary to conventional techniques represented by biochemical markers, alternative methods using miRNA, like microarrays and qRT-PCR, can detect early hepatic injury. In this context, it is important to reiterate that histopathological examination is a slow process (Yudate et al. 2012). Thus, the use of such techniques has been stimulated because they detect changes even before the lesions appear in the liver cells (Zhang et al. 2010). Further, the changes in concentrations of miRNA in the tissue and plasma including those

like miR-122, miR-192, miR-193, miR-710, and miR-711 may precede the onset of the histopathological changes in the hepatocytes (Su et al. 2012; Zhang et al. 2010; Wang et al. 2009).

In animal models, the differentiated expression pattern of the miRNA assessed at development stages of NAFLD, steatosis, and steatohepatitis indicates the possibility of using such a tool for diagnosis, because it is able to discriminate the distinct stages of the diseases differentially expressed by the miRNA (Jin et al. 2009). During phospholipidosis it was observed that the pathology induced by the drugs is preceded by a characteristic gene expression pattern. Interestingly, this pattern can already be detected 24 h after drug administration (Yudate et al. 2012).

The hepatic expression of miR-33a and miR-122 in obese HFD-fed rats was normalized after 3 weeks' consumption of dietary proanthocyanidins from grape seed. The results of this study showed that supplementation with a proanthocyanidin extract was able to suppress the miRNA overexpression in all the doses tested, which indicates that the lower doses of the extract were sufficient to normalize the miRNA changes induced by the HFD (Baselga-Escudero et al. 2015).

Besides, a dietary intervention study with vitamin E showed that the daily ingestion of multiple doses of this vitamin can affect the liver miRNA concentrations. Thus, rats fed with a vitamin E-deficient diet revealed lower miRNA concentrations (miR-122a and miR-125b) when compared with the animals receiving the vitamin for 6 months. Such studies are of great interest, as this vitamin plays a crucial role in lipid metabolism and inflammation, which are usually involved in the development of liver disease. Nevertheless, nutritional intervention studies using miRNA as a marker for liver damage assessment are still limited thus far (Gaedicke et al. 2008).

The use of miRNA as biomarkers is noninvasive and presents stability in the body fluids. Furthermore, the modulation of some specific miRNA is being regarded as a promising strategy to treat metabolic diseases (Baselga-Escudero et al. 2015).

Conclusion

The awareness of new methodologies along with the biomarkers used in the diagnosis or detection of changes involved in liver diseases plays a significant role in identifying hepatic damage.

Studies using biochemical markers have been regarded as good predictors to estimate the changes occurring in liver diseases. Besides, cytohistological analysis is able to detect visible liver changes. Further, these are useful tools to assess the in vivo effects of the bioactive compounds. Moreover, the nutrigenomic approach has been used to evaluate the effect of nutritional interventions on the genes, proteins, and metabolic changes in chronic liver diseases, thus contributing toward improvement in the prognostic in liver pathogenesis. However, as these biomarkers are complementary, more than one is necessary to accurately evaluate liver damage.

Nutritional intervention studies have shown promising results regarding the prevention and even treatment of liver damage, especially NAFLD. In general,

these positive results are a consequence of the antioxidant and anti-inflammatory activities arising from the bioactive compounds naturally occurring in foods.

Potential Applications to Prognosis, Other Diseases, or Conditions

Nutritional intervention using bioactive compounds, prebiotics, probiotics, and nutrients may represent a novel therapeutic approach to prevent or attenuate diet-related disease. Therefore, healthy diets may protect against metabolic diseases, such as carcinogenesis, obesity, diabetes, and cardiovascular diseases, in view of their ability to exert antioxidant, anti-inflammatory, anti-lipidemic activities, and improving the intestinal barrier.

Biomarkers are used to evaluate the nutritional interventional effects on understanding the spectrum of metabolic diseases, being used for screening, staging determination, and therapy monitoring. Advances in nutrigenomic have generated many candidate biomarkers which allow the individualization of treatment improving the disease prognostic.

Summary Points

- Nutritional intervention with probiotic or phenolic compound-rich food can modulate biochemical markers and regulate the oxidative balance.
- Functional foods can influence the liver inflammation.
- Cytohistological analysis is able to detect visible liver abnormalities.
- Nutrigenomic approaches are a good tool to evaluate the effect of nutritional interventions in chronic liver diseases using animal models.
- The use of micro-RNAs as biomarkers is noninvasive and presents stability in body fluids.

References

Abenavoli L, Peta V. Role of adipokines and cytokines in non-alcoholic fatty liver disease. Rev Recent Clin Trials. 2014;9:134–40.

Afman L, Muller M. Nutrigenomics: from molecular nutrition to prevention of disease. J Am Diet Assoc. 2006;106:569–76.

Bahcecioglu IH, Kuzu N, Metin K, et al. Lycopene prevents development of steatohepatitis in experimental nonalcoholic steatohepatitis model induced by high-fat diet. Vet Med Int. 2010;2010:1–8.

Baselga-Escudero L, Pascual-Serrano A, Ribas-Latre A, et al. Long-term supplementation with a low dose of proanthocyanidins normalized liver miR-33a and miR-122 levels in high-fat diet–induced obese rats. Nutr Res. 2015;35:337–45.

Behne T, Copur MS. Biomarkers for hepatocellular carcinoma. Int J Hepatol. 2012;2012:859076.

Berg AH, Combs TP, Scherer PE. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. Trends Endocrinol Metab. 2002;13:84–9.
Bernal B, Martín-Pozuelo G, Lozano AB, Sevilla C, García-Alonso J, Canovas M, Periago MJ. Lipid biomarkers and metabolic effects of lycopene from tomato juice on liver of rats with induced hepatic steatosis. J Nutr Biochem. 2013;24:1870–81.
Bhathena J, Martoni C, Kulamarva A, Tomaro-Duchesneau C, Malhotra M, Paul A, Urbanska AM, Prakash S. Oral probiotic microcapsule formulation ameliorates non-alcoholic fatty liver disease in Bio F1 B golden syrian hamsters. PLoS One. 2013;8, e58394.
Bouchard C. Gene-environment interactions in the etiology of obesity: defining the fundamentals. Obesity (Silver Spring). 2008;16:S5–10.
Boyd SD. Everything you wanted to know about small RNA but were afraid to ask. Lab Invest. 2008;88:569–78.
Brun-Heath I, Rmonval M, Chabrol E, Xiao J, Palkovits M, Lyck R, Miller F, Couraud PO, Mornet E, Fonta C. Differential expression of the bone and the liver tissue non – specific alkaline phosphatase isoforms in brain tissues. Cell Tissue Res. 2011;343:521–36.
Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck A, Lambert DM, Muccioli GG, Delzenne NM. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009;58:1091–103.
Cao W, Zhou Y, Li Y, Zhang XR, He M, Zang N, Zhou Y, Liao M. iTRAQ-based proteomic analysis of combination therapy with taurine, epigallocatechin gallate, and genistein on carbon tetrachloride-induced liver fibrosis in rats. Toxicol Lett. 2015;232:233–45.
Cesaro C, Tiso A, Prete AD, Cariello R, Tuccillo C, Cotticelli G, Blanco CV, Loguercio C. Gut microbiota and probiotics in chronic liver diseases. Dig Liver Dis. 2011;43:431–8.
Chalabi N, Bernard-Gallon DJ, Vasson MP, et al. Nutrigenomics and antioxidants. Pers Med. 2008;5:25–36.
Chang CC, Chang CY, Huang JP, et al. Effect of resveratrol on oxidative and inflammatory stress in liver and spleen of streptozotocin-induced type 1 diabetic rats. Chin J Physiol. 2012;55:192–201.
Chen JY, Chou HC, Chen YH, et al. High glucose-induced proteome alterations in hepatocytes and its possible relevance to diabetic liver disease. J Nutr Biochem. 2013;24:1889–910.
Chung APYS, Ton SH, Gurtu S, et al. Ellagitannin geraniin supplementation ameliorates metabolic risks in high-fat diet-induced obese Sprague Dawley rats. J Funct Foods. 2014;9:173–82.
Cordero P, Campion J, Milagro FI, et al. Transcriptomic and epigenetic changes in early liver steatosis associated to obesity: effect of dietary methyl donor supplementation. Mol Genet Metab. 2013;110:388–95.
Cowan ML, Rahman TM, Krishna S. Proteomic approaches in the search for biomarkers of liver fibrosis. Trends Mol Med. 2010;16:171–83.
D'Argenio G, Amoruso DC, Mazzone G, et al. Garlic extract prevents CCl(4)-induced liver fibrosis in rats: the role of tissue transglutaminase. Dig Liver Dis. 2010;42:571–7.
Davis CD, Milner J. Frontiers in nutrigenomics, proteomics, metabolomics and cancer prevention. Mutat Res. 2004;551:51–64.
DeBusk R. Diet-related disease, nutritional genomics, and food and nutrition professionals. J Am Diet Assoc. 2009;109:410–3.
Elliott R, Ong TJ. Nutritional genomics. BMJ. 2002;324:1438–42.
Endo H, Niioka M, Kobayashi N, et al. Butyrate-producing probiotics reduce nonalcoholic fatty liver disease progression in rats: new insight into the probiotics for the gut-liver axis. PLoS One. 2013;8, e63388.
Eslamparast T, Eghtesad S, Poustchi H, et al. Recent advances in dietary supplementation, in treating non-alcoholic fatty liver disease. World J Hepatol. 2015;7:204–12.
Esposito E, Iacono A, Bianco G, et al. Probiotics reduce the inflammatory response induced by a high-fat diet in the liver of young rats. J Nutr. 2009;139:905–11.

Everard A, Matamoros S, Geurts L, et al. Saccharomyces boulardii administration changes gut microbiota and reduces hepatic steatosis, low-grade inflammation, and fat mass in obese and type 2 diabetic db/db mice. MBio. 2014;5:e01011–4.

FAO/WHO. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Food and Agriculture Organization of the United Nations. Córdoba, Argentina 2001.

Ferolla SM, Armiliato GNA, Couto CA, et al. The role of intestinal bacteria overgrowth in obesity-related nonalcoholic fatty liver disease. Nutrients. 2014;6:5583–99.

Gaedicke S, Zhang X, Schmelzer C, et al. Vitamin E dependent microRNA regulation in rat liver. FEBS Lett. 2008;582:3542–6.

Ganai AA, Khan AA, Malik ZA, et al. Genistein modulates the expression of NF-kappaB and MAPK (p-38 and ERK1/2), thereby attenuating d-Galactosamine induced fulminant hepatic failure in Wistar rats. Toxicol Appl Pharmacol. 2015;283:139–46.

Gasparotto J, Somensi N, Bortolin RC, et al. Preventive supplementation with fresh and preserved peach attenuates CCl4-induced oxidative stress, inflammation and tissue damage. J Nutr Biochem. 2014;25:1282–95.

Jia XY, Zhang QA, Zhang ZQ, et al. Hepatoprotective effects of almond oil against carbon tetrachloride induced liver injury in rats. Food Chem. 2011;125:673–8.

Jia HJ, Saito KJ, Aw WP, et al. Transcriptional profiling in rats and an ex vivo analysis implicate novel beneficial function of egg shell membrane in liver fibrosis'. J Funct Foods. 2013;5:1611–9.

Jin X, Ye YF, Chen SH, et al. MicroRNA expression pattern in different stages of nonalcoholic fatty liver disease. Dig Liver Dis. 2009;41:289–97.

Kang JH, Goto T, Han IS, et al. Dietary capsaicin reduces obesity-induced insulin resistance and hepatic steatosis in obese mice fed a high-fat diet. Obesity (Silver Spring). 2010; 18:780–7.

Kannappan S, Palanisamy N, Anuradha CV. Suppression of hepatic oxidative events and regulation of eNOS expression in the liver by naringenin in fructose-administered rats. Eur J Pharmacol. 2010;645:177–84.

Kuzu N, Metin K, Dagli AF, et al. Protective role of genistein in acute liver damage induced by carbon tetrachloride. Mediators Inflamm. 2007;2007:36381.

Li YY. Genetic and epigenetic variants influencing the development of nonalcoholic fatty liver disease. World J Gastroenterol. 2012;18:6546–51.

Liu LF, Purushotham A, Wendel AA, et al. Combined effects of rosiglitazone and conjugated linoleic acid on adiposity, insulin sensitivity, and hepatic steatosis in high-fat-fed mice. Am J Physiol Gastrointest Liver Physiol. 2007;292:G1671–82.

Lo RS, Austin AS, Freeman JG. Is there a role for probiotics in liver disease? Sci World J. 2014;8:747–68.

Mallat A, Lotersztajn S. Cellular mechanisms of tissue fibrosis. 5. Novel insights into liver fibrosis. Am J Physiol Cell Physiol. 2013;305:C789–99.

Manach C, Scalbert A, Morand C, et al. Polyphenols: food sources and bioavailability. Am J Clin Nutr. 2004;79:727–47.

Mann JI. Nutrition recommendations for the treatment and prevention of type 2 diabetes and the metabolic syndrome: an evidenced-based review. Nutr Rev. 2006;64:422–7.

Mbimba T, Awale P, Bhatia D, et al. Alteration of hepatic proinflammatory cytokines is involved in the resveratrol-mediated chemoprevention of chemically-induced hepatocarcinogenesis'. Curr Pharm Biotechnol. 2012;13:229–34.

Miura K, Ohnishi H. Role of gut microbiota and Toll-like receptors in nonalcoholic fatty liver disease. World J Gastroenterol. 2014;20:7381–91.

Muller M, Kersten S. Nutrigenomics: goals and strategies. Nat Rev Genet. 2003;4:315–22.

Nardone G, Compare D, Liguori E, et al. Protective effects of Lactobacillus paracasei F19 in a rat model of oxidative and metabolic hepatic injury. Am J Physiol Gastrointest Liver Physiol. 2010;299:G669–76.

Neyrinck AM, Possemiers S, Verstraete W, et al. Dietary modulation of clostridial cluster XIVa gut bacteria (Roseburia spp.) by chitin-glucan fiber improves host metabolic alterations induced by high-fat diet in mice. J Nutr Biochem. 2012;23:51–9.
Ning C, Liu L, Lv G, et al. Lipid metabolism and inflammation modulated by Vitamin D in liver of diabetic rats. Lipids Health Dis. 2015;14:31.
Noll C, Raaf L, Planque C, et al. Protection and reversal of hepatic fibrosis by red wine polyphenols in hyperhomocysteinemic mice. J Nutr Biochem. 2011;22:856–64.
Novello AA, Conceição LL, Dias MMS, et al. Chemical characterization, antioxidant and antiatherogenic activity of anthocyanin-rich extract from Euterpe edulis Mart. in mice. J Food Nutr Res. 2015;54:101–112
Nugent C, Younossi ZM. Evaluation and management of obesity-related nonalcoholic fatty liver disease. Nat Clin Pract Gastroenterol Hepatol. 2007;4:432–41.
Oliveira TB, Rogero MM, Genovese MI. Poliphenolic-rich extracts from cocoa (Theobroma cacao L.) and cupuassu (Theobroma grandiflorum Willd. Ex Spreng. K. Shum) liquors: a comparison of metabolic effects in high-fat fed rats. PharmaNutrition. 2015;3:20–8.
Ong JP, Elariny H, Collantes R, et al. Predictors of nonalcoholic steatohepatitis and advanced fibrosis in morbidly obese patients. Obes Surg. 2005;15:310–5.
Ozer J, Ratner M, Shawc M, et al. The current state of serum biomarkers of hepatotoxicity. Toxicol Lett. 2008;245:194–205.
Pan MH, Lai CS, Tsai ML, et al. Chemoprevention of nonalcoholic fatty liver disease by dietary natural compounds'. Mol Nutr Food Res. 2014;58:147–71.
Park HJ, Lee JY, Chung MY, et al. Green tea extract suppresses NFkappaB activation and inflammatory responses in diet-induced obese rats with nonalcoholic steatohepatitis. J Nutr. 2012;142:57–63.
Park HJ, Jung UJ, Cho SJ, et al. Citrus unshiu peel extract ameliorates hyperglycemia and hepatic steatosis by altering inflammation and hepatic glucose- and lipid-regulating enzymes in db/db mice. J Nutr Biochem. 2013;24:419–27.
Parnell JA, Raman M, Rioux KP, et al. The potential role of prebiotic fibre for treatment and management of non-alcoholic fatty liver disease and associated obesity and insulin resistance. Liver Int. 2012;32:701–11.
Plaza-Diaz J, Gomez-Llorente C, Abadia-Molina F, et al. Effects of Lactobacillus paracasei CNCM I-4034, Bifidobacterium breve CNCM I-4035 and Lactobacillus rhamnosus CNCM I-4036 on hepatic steatosis in Zucker rats. PLoS One. 2014;9, e98401.
Rahman I, Biswas SK, Kirkham PA. Regulation of inflammation and redox signaling by dietary polyphenols. Biochem Pharmacol. 2006;72:1439–52.
Ramaiah SK. A toxicologist guide to the diagnostic interpretation of hepatic biochemical parameters. Food Chem Toxicol. 2007;45:1551–7.
Ramirez-Torres A, Barcelo-Batllori S, Fernandez-Vizarra E, et al. Proteomics and gene expression analyses of mitochondria from squalene-treated apoE-deficient mice identify short-chain specific acyl-CoA dehydrogenase changes associated with fatty liver amelioration. J Proteomics. 2012;75:2563–75.
Ramirez-Tortosa MC, Ramirez-Tortosa CL, Mesa MD, et al. Curcumin ameliorates rabbits's steatohepatitis via respiratory chain, oxidative stress, and TNF-alpha. Free Radic Biol Med. 2009;47:924–31.
Rao AV, Rao LG. Carotenoids and human health. Pharmacol Res. 2007;55:207–16.
Riserus U. Fatty acids and insulin sensitivity. Curr Opin Clin Nutr Metab Care. 2008;11:100–5.
Rodriguez-Suarez E, Gonzalez E, Hughes C, et al. Quantitative proteomic analysis of hepatocyte-secreted extracellular vesicles reveals candidate markers for liver toxicity. J Proteomics. 2014;103:227–40.
Salih SM, Nallasamy P, Muniyandi P, et al. Genistein improves liver function and attenuates non-alcoholic fatty liver disease in a rat model of insulin resistance. J Diabetes. 2009;1:278–87.
Savcheniuk O, Kobyliak N, Kondro M, et al. Short-term periodic consumption of multiprobiotic from childhood improves insulin sensitivity, prevents development of non-alcoholic fatty liver

disease and adiposity in adult rats with glutamate-induced obesity. BMC Complement Altern Med. 2014;16;14:247

Sekhon-Loodu S, Catalli A, Kulka, et al. Apple flavonols and n-3 polyunsaturated fatty acid-rich fish oil lowers blood C-reactive protein in rats with hypercholesterolemia and acute inflammation. Nutr Res. 2014;34:535–43.

Siddeek B, Inoubli L, Lakhdari N, et al. MicroRNAs as potential biomarkers in diseases and toxicology. Mutat Res. 2014;764–765:46–57.

Su Y-W, Chen X, Jiang Z-Z, et al. A panel of serum MicroRNAs as specific biomarkers for diagnosis of compound- and herb-induced liver injury in rats'. PLoS One. 2012;7, e37395.

Tilg H, Kaser A, Moschen AR. How to modulate inflammatory cytokines in liver diseases. Liver Int. 2006;26:1029–39.

Tsao R. Chemistry and biochemistry of dietary polyphenols. Nutrients. 2010;2:1231–46.

Wang J-Q, Li J, Zou YH, et al. Preventive effects of total flavonoids of Litsea coreana leve on hepatic steatosis in rats fed with high fat diet. J Ethnopharmacol. 2009a;121:54–60.

Wang K, Zhang S, Marzolf B, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc Natl Acad Sci. 2009b; vol. 106, p. 4402–4407.

Wang Y, Ausman LM, Greenberg AS, et al. Dietary lycopene and tomato extract supplementations inhibit nonalcoholic steatohepatitis-promoted hepatocarcinogenesis in rats. Int J Cancer. 2010;126:1788–96.

Wang L, Meng X, Zhang F. Raspberry ketone protects rats fed high-fat diets against nonalcoholic steatohepatitis. J Med Food. 2012;15:495–503.

Wang H, Sit WH, Tipoe GL, Wan JM. Differential protective effects of extra virgin olive oil and corn oil in liver injury: a proteomic study. Food Chem Toxicol. 2014;74:131–8.

Weerawatanakorn M, Hsieh SC, Tsai ML, et al. Inhibitory effect of tetrahydrocurcumin on dimethylnitrosamine-induced liver fibrosis in rats. J Funct Foods. 2014;7:305–13.

Wieckowska A, Mccullough AJ, Feldstein AE. Noninvasive diagnosis and monitoring of nonalcoholic steatohepatitis: present and future. Hepatology. 2007;46:582–9.

Yeh YH, Hsieh YL, Lee YT. Effects of yam peel extract against carbon tetrachloride-induced hepatotoxicity in rats. J Agric Food Chem. 2013;61:7387–96.

Yokoi T, Nakajima M. microRNAs as mediators of drug toxicity. Annu Rev Pharmacol Toxicol. 2013;53:377–400.

Yokota T, Oritani K, Takahashi I, et al. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. Blood. 2000;96:1723–32.

Younossi ZM, Baranova A, Ziegler K, et al. A genomic and proteomic study of the spectrum of nonalcoholic fatty liver disease. Hepatology. 2005;42:665–74.

Yudate HT, Kai T, Aoki M, et al. Identification of a novel set of biomarkers for evaluating phospholipidosis-inducing potential of compounds using rat liver microarray data measured 24-h after single dose administration. Toxicology. 2012;295:1–7.

Zhang Y, Jia Y, Zheng R, et al. Plasma MicroRNA-122 as a biomarker for viral, alcohol-, and chemical-related hepatic diseases. Clin Chem. 2010;56:1830–8.

Serum Biomarkers for Evaluating Portal Hypertension

7

Saad Elias, Barhoum Masad, and Assy Nimer

Contents

Abstract

Cirrhosis represents the final stage for wide variety of chronic liver diseases, regardless of its etiology, and the development of portal hypertension is responsible for the pathogenesis of most frequent and fatal complications of cirrhosis. It is of most importance to evaluate patients newly diagnosed with cirrhosis for the presence of clinically significant portal hypertension and associated complications, which could expose the patient to fatal conditions such as variceal bleeding. The most accurate method for evaluating the presence and severity of portal hypertension is the measurement of the hepatic venous pressure gradient, which

S. Elias (✉) • A. Nimer (✉)
Department of Internal Medicine A, Galilee Medical Center, Nahariya, Israel

Bar Ilan University, Safed, Israel
e-mail: eliass@gmc.gov.il; nimera@gmc.gov.il

B. Masad
Bar Ilan University, Safed, Israel

Galilee Medical Center, Nahariya, Israel

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_33

on one hand provides us valuable prognostic information but on the other hand represents a problematic technique, because it is invasive, costly, and not available in all centers. Several alternative noninvasive techniques have been proposed to assess portal hypertension, including serum biomarkers and imaging techniques. Various serum molecules have been investigated for their ability to predict the presence of portal hypertension, some of which have showed to either correlate with the hepatic venous pressure gradient or predict clinically significant portal hypertension. This chapter will focus on the potential role of multiple serum markers of portal hypertension that could be clinically applied to predict the presence of clinically significant portal hypertension, to stratify patients with respect to the severity of portal hypertension, to predict lethal complications such as variceal bleeding, and to monitor disease progression or treatment response without exposing patients to the risks of repeated invasive assessment.

Keywords
Apelin • Asymmetric dimethylarginine • Child-Pugh score • Cholestasis • Cirrhosis • Dimethylargininedimethylaminohydrolase-1 (DDAH-1) • Endothelial dysfunction • Esophageal varices • Fibrotest • Hepatic encephalopathy

Key Facts of Portal Hypertension

The key facts of portal hypertension including the function of liver, blood supply of liver, the pathogenesis of cirrhosis associated portal hypertension, causes of portal hypertension, and principles of treatment are listed below:

- The liver is a vital organ that has numerous functions in the human body, including metabolism of lipids, carbohydrates, amino acids and serum proteins; hormone production; production of bile, which is necessary for digestion; and detoxification.
- The liver receives a dual blood supply from the portal vein and hepatic arteries. The portal system includes all veins that carry blood from the abdominal part of the alimentary tract, the spleen, pancreas, and gallbladder.
- Chronic liver disease is characterized by damage and regeneration of liver parenchyma leading to liver fibrosis and cirrhosis
- Liver cirrhosis is characterized by two pathologic conditions: the first is loss of function of the liver, and the second is increased resistance to portal flow and elevation of the pressure in the portal system.
- The increased resistance to portal flow is either mechanical due to the disturbed architecture and nodularity of cirrhosis or dynamic due to dysfunction of the endothelium and reduced bioavailability of nitric oxide (NO).
- This increased pressure in the portal vein may lead to the development of large, swollen veins (varices) within the esophagus, stomach, rectum, or umbilical area. Varices can rupture and bleed, resulting in potentially life-threatening complications.

- Causes of portal hypertension other than cirrhosis include suprahepatic causes, such as thrombosis of inferior vena cava or hepatic vein and cardiac diseases (constrictive pericarditis for example), and infrahepatic causes such as portal vein thrombosis.
- The treatment of portal hypertension is mainly supportive and consists of managing portal hypertension related complications, and the only way to fully cure portal hypertension is liver transplantation

Definitions of Words and Terms

Ascites	A term that describes the accumulation of more than 25 ml of fluid in the peritoneal cavity.
Cholestasis	A condition that describes an impaired bile formation and flow. The causes are classified as intrahepatic, in which there is a secretory defect of the hepatocytes and cholangiocytes, and extrahepatic characterized by obstruction of bile ducts.
Cirrhosis	An abnormal condition of liver characterized by development of scar tissue that replaces normal liver tissue leading to decreased in hepatocellular mass, and thus decreased liver function, and an alteration of blood flow with an increased pressure in the blood vessels that supplies the liver.
Endothelial dysfunction	A pathologic state of the function of endothelium, the cellular inner lining of blood vessels, in which its ability to regulate the vascular tone is decreased.
Esophageal varices (EV)	Varices are abnormally dilated blood vessels, usually veins. In patients with cirrhosis, an elevation in blood pressure in the portal vein leads to the formation of varices in multiple sites in the abdomen, but the most important is the lower third of esophagus.
Extracellular matrix (ECM)	ECM is the noncellular component present within all tissues and organs, composed of proteins and polysaccharides that are secreted locally, and their function is to provide support, segregate tissues from one another, and regulate intercellular communication.
Hepatic encephalopathy (HE)	A syndrome observed in patients with liver cirrhosis, characterized by personality changes, intellectual impairment, and a depressed level of consciousness.
Hepatorenal syndrome (HRS)	HRS is a syndrome of progressive kidney injury seen in patients with liver cirrhosis and portal hypertension and associated with high risk of motality.
Insulin resistance	A condition in which the insulin, a hormone that regulates carbohydrate and lipid metabolism, has a

	decreased activity on its receptors in insulin-sensitive tissues such as liver, skeletal muscle, and adipose tissues.
The Model for End-stage Liver Disease (MELD)	MELD is a scoring system for assessing the severity of chronic liver disease. The score uses a patient's laboratory values for serum bilirubin, serum creatinine, and the international normalized ratio (INR) for prothrombin time to predict 3-month survival.
Spontaneous bacterial peritonitis (SBP)	SBP is an acute bacterial infection of ascitic fluid that complicates patients with liver cirrhosis, diagnosed when the peritoneal fluid contains excess of neutrophils of more than 250 cells per mm^3. SBP is associated with high rate of mortality.
Thrombopoietin (TPO)	TPO is a hormone produced by liver, and its function is to stimulate platelet production by bone marrow.

Introduction

Development of clinically significant portal hypertension (CSPH) is a major cornerstone in the natural history of any chronic liver disease (CLD) regardless of the etiologic cause of CLD, and it is associated with clinical decompensation and development of portal hypertension (PTH)-related complications. Portal hypertension is defined as a pathological increase in portal venous pressure between the portal vein and the inferior vena cava to higher than the normal range ($\leq$5 mmHg).

The clinical manifestations of portal hypertension include ascites, gastroesophageal varices, hepatic encephalopathy (HE), variceal bleeding, spontaneous bacterial peritonitis (SBP), infections other than SBP, and hepato-renal syndrome (HRS). The occurrence of these complications reflects the severity of portal hypertension and substantially worsens the prognosis of cirrhosis.

Patients with cirrhosis are considered high risk if they experience any episode of decompensation associated with high mortality risk such as variceal bleeding, refractory ascites, SBP, HRS, hyponatremia, and HE. The role of a prognostic marker, at this stage, is to help in identifying, among patients with decompensation, those at the highest mortality risk in order to implement more aggressive therapeutic strategies.

One of the most accurate methods for determining portal venous pressure involves the catheterization of the hepatic vein and the measurement of the hepatic venous pressure gradient (HVPG) defined as the difference between the wedged or occluded hepatic venous pressure and the free hepatic venous pressure (Lebrec et al. 1997) and if measured precisely, has a very low variability. Portal hypertension is considered moderate when the HVPG ranges from 5 to 10 mmHg and severe when the HVPG is greater than 10 mmHg. HVPG correlates with both structural and functional changes that occur in cirrhosis, and it carries valuable prognostic information to stratify the mortality risk. CSPH is established when HVPG is $>$ 10 mmHg, and at this value the

patient usually develops of PTH-related complications, while if HVPG is > 12 mmHg, the risk of variceal bleeding increases (Bosch et al. 2009). Some meta-analyses have demonstrated that a reduction of HVPG to < 12 mmHg or more than 20% of baseline significantly reduces the risk of bleeding (Feu et al. 1995; Merkel et al. 2000; Bureau et al. 2002), whereas HVPG values equal to or below 8 mmHg are expected to control refractory ascites (Sanyal et al. 2003). Several studies demonstrated that HVPG has an independent predictive value on mortality in decompensated patients (D'Amico et al. 2006). Ripoll et al. (2005) in a series of 393 patients, mostly with previous decompensation, showed that the HVPG, independently of the model for end-stage liver disease (MELD) score, had an overall effect of 3% increase of mortality for each 1 mmHg of HVPG increase.

Despite its excellent diagnostic and prognostic value, the use of HVPG in clinical practice is limited due to several factors such as the invasiveness of the procedure, availability only in specialized centers, and excessive costs (Merkel and Montagnese 2011).

Acute variceal bleeding is the complication of advanced cirrhosis with the highest mortality reduction achieved in the last decades, from 40%–50% to 10%–20% (Chalasani et al. 2003). In acute variceal bleeding, an HVPG ≥ 20 mmHg identifies patients at high risk of early rebleeding and bleeding-related mortality (Moitinho et al. 1999). Upper Gastrointestinal tract endoscopy is the gold-standard technique for identifying esophageal and gastric varices and is essential for the endoscopic management of variceal hemorrhage (Garcia-Tsao et al. 2007). There is consensus that it is mandatory to screen for EV by endoscopy when the diagnosis of cirrhosis is established, and the endoscopy should be repeated at 2–3 years interval in patients without varices. However, this approach has some limitations, as endscopy is an invasive procedure; the cost-effectiveness is questionable because only 9–36% of patients with cirrhosis found to have varices on screening endoscopy.

Currently, there is no established noninvasive test that can predict portal pressure among patients with chronic liver disease, and the ability to predict portal pressure with a simple blood test would revolutionize clinical management of patients with chronic liver disease.

Serum biomarkers of portal hypertension can be classified to parameters that reflect liver function, markers related to complications of portal hypertension, markers related to the pathogenesis of portal hypertension, and models combining multiple parameters.

Markers Related to Liver Function

Liver functions tests can be classified into parameters related to cell lysis or inflammation (AST and ALT), parameters of cholestasis (γGT and bilirubin), and parameters that reflect hepatocyte synthetic function (PT-INR, albumin). These parameters are surrogates of inflammation and steatosis, which have a significant predictive value for the progression of fibrosis (Gordon et al. 2005).

Child-Pugh score and its objective component (albumin, bilirubin, INR) correlate with HVPG (Braillon et al. 1986; Gluud et al. 1988; Stanley et al. 1998) and correlate with the prevalence and grade of esophageal varices in cirrhotic patients. Interestingly, this correlation is observed also in patients with compensated cirrhosis (Berzigotti et al. 2008), suggesting that a close relationship exists between the structural changes which give onset to portal hypertension and hepatocellular dysfunction. Another model obtained by the combination of biochemical parameters, namely, albumin, ALT, and INR, had an area under the curve (AUROC) of 0.952 in the prediction of CSPH (Berzigotti et al. 2008).

Markers Related to Complications of Portal Hypertension

Platelet Count

Thrombocytopenia, defined as platelet counts $<$150,000/μL, is a common complication in patients with chronic liver disease (CLD), reported in as many as 76% of cirrhotic patients (Giannini 2006). The major mechanisms for thrombocytopenia in liver cirrhosis include the portal hypertension related hypersplenism leading to platelet sequestration in the spleen and the decreased production of platelets in the bone marrow due to decreased production of thrombopoietin in the liver. The degree of thrombocytopenia also correlates with the extent of chronic hepatic injury. Studies have shown that platelet count is one of the factors that reflect the degree of liver fibrosis or the severity of liver cirrhosis (Wai et al. 2003, 2006). Studies have also showed a correlation between platelet count and HVPG and that thrombocytopenia can predict the presence of esophageal varices. One study showed that it was approximately five times more likely for large esophageal varices or gastric varices to be present if the platelet count was $<$88,000 and the negative predictive value for large esophageal varices was 92% (Zaman et al. 1999).

The Aspartate Aminotransferase/Platelet Ratio Index (APRI)

The APRI, calculated as AST (U/L)/ upper limit of normal $\times$ 100/platelet count (109/L), was first introduced by Wai et al. in 2003 as a simple noninvasive tool for the diagnosis of significant fibrosis and cirrhosis of various etiologies (Shin et al. 2007, 2008; Forestier et al. 2010). Subsequently many studies have shown the APRI correlates with HVPG, and an APRI of $\geq$ 1.09 had a sensitivity 66%, specificity 73%, positive predictive value 85%, negative predictive value 47%, and diagnostic accuracy 68% for predicting HVPG $>$12 mmHg.

Serum Ascites Albumin Gradient (SAAG)

SAAG, which was first proposed by Hoefs et al. in 1981 (Hoefs 1983), is calculated by subtracting the ascites albumin concentration from the serum albumin concentration. The SAAG has replaced total ascetic protein in evaluating the causal mechanism of ascites, because studies have shown that a high SAAG ($\geq$1.1 g/dL) predicts portal hypertension with accuracy rate of 97% and sensitivity of 100% (Table 1).

Table 1 Serum biomarkers for evaluating portal hypertension. This table represents a simple classification of blood test that can be used as serum biomarkers of portal hypertension, and it lists four groups of markers, which are widely discussed within the article body

Markers related to liver function
Markers related to cell lysis or inflammation
Markers of cholestasis
Markers that reflect hepatocyte synthetic function
Markers related to complications of portal hypertension
Parameters that represent hypersplenism
Parameters defining the cause of ascites
Markers related to the pathogenesis of portal hypertension
Markers of endothelial function
Parameters that assess hepatic fibrosis
Markers of inflammation
Metabolic markers
Markers that represents models combining multiple parameters

Markers Related to the Pathogenesis of Portal Hypertension

Markers of Endothelial Function

Endothelial dysfunction is a major determinant of the increased intrahepatic vascular tone observed in cirrhosis, and a number of markers reflecting this dysfunction have been identified.

Von Willebrand factor antigen (VWF-Ag) is a large adhesive protein released by activated endothelial cells and therefore represents an indicator of endothelial cell activation (Van Mourik et al. 1999), and it is used as a surrogate marker of endothelial dysfunction (Lavi et al. 2008). Endothelial dysfunction is an early key event in many vascular diseases and is considered a major determinant of the increased hepatic vascular tone of cirrhotic liver (Iwakiri and Groszmann 2007). Levels of VWF are increased in patients with cirrhosis and correlate with the grade of fibrosis and the severity of liver disease (Lisman et al. 2002). Studies have shown that VWF-Ag level correlated with HVPG and high levels of VWF-Ag is associated with CSPH, the presence of esophageal varices, and increased risk of mortality (La Mura et al. 2011; Ferlitsch et al. 2012). The AUC for the detection of CSPH using a VWF-Ag cut-off value of $\geq$ 241% is 0.85.

Recently, the VITRO Score (the Von Willebrand factor-Ag/thrombocyte ratio) was introduced as a marker of cirrhosis and portal hypertension (Hametner et al. 2016). The VITRO score was significantly higher in patients with CSPH compared to patients with HVPG <10 mmHG (median 3.21 versus 1.29; <0.0001), it was also higher in patients with esophageal varices ($P < 0.0001$) and ascites ($P < 0.014$). The diagnostic accuracy of the VITRO score for detecting CSPH shows an AUC of 0.86 (CI 0.81–0.91) with a sensitivity of 80% and a specificity of 70% at a cut-off >1.58. The correlation between CSPH and the VITRO score was independent of Child-Pugh score. This score should be validated in further studies.

Nitric oxide (NO) is an essential regulator of intrahepatic vascular tone. In cirrhosis, the hepatic NO levels are significantly reduced, with associated

elevated sinusoidal vascular resistance (Iwakiri et al. 2008). NO synthesis by endothelial nitric oxide synthase (eNOS) can be inhibited by the competitive endogenous inhibitor asymmetric dimethylarginine (ADMA). ADMA is metabolized in the liver, thus impaired liver function is associated with increased plasma levels of ADMA. Lluch and coworkers showed that peripheral blood levels of ADMA correlated with the degree of liver failure and decompensation in patients with alcohol-related cirrhosis (Lluch et al. 2004). In a further study involving patients with compensated chronic hepatitis C cirrhosis, a positive statistically significant correlation was found between HVPG and ADMA (Vizzutti et al. 2007).

Recently, a study using animal model of cirrhosis and portal hypertension demonstrated that Dimethylargininedimethylaminohydrolase-1 (DDAH-1), which is the key enzyme metabolizing hepatic ADMA, is a specific molecular target for portal pressure reduction, through actions on ADMA-mediated regulation of eNOS activity (Mookerjee et al. 2015).

Further studies are needed to define ADMA metabolism and function in Portal hypertension and its ability to predict CSPH.

Apelin is an endogenous ligand for angiotensin-like receptor 1, and it is distributed across numerous organs, including the brain, liver, heart, spleen, kidney, and lung. In several preclinical studies with cirrhotic animal model, serum levels of apelin (s-apelin) showed close relationships with both intrahepatic fibrosis and splanchnic hemodynamics. Its clinical utility as a biomarker of portal hypertension and prognosis was recently investigated (Lim et al. 2016). s-apelin had a direct correlation with the degree of hepatic fibrosis and it also showed a significant linear correlation with HVPG ($R2 = 0.356$, $P < 0.001$). The diagnostic ability of s-apelin for CSPH was also better than traditional prognosis marker Child-Pugh score and MELD score.

Markers of Hepatic Fibrosis

In advanced stages of fibrosis, the liver contains around six to eight times more extracellular matrix (ECM) proteins than the normal liver (Schuppan et al. 2001; Weiler-Normann et al. 2007). ECM mainly consists of types I, III, and IV collagen, fibronectin, laminin, hyaluronan, elastin, undulin, and proteoglycan (Gressner and Weiskirchen 2006; Baranova et al. 2011; Bataller and Brenner 2005). The proteins are found in the blood and their level correlate with the development of hepatic fibrosis. These proteins were also studied as markers for predicting severe PHT. Serum laminin levels were shown to significantly correlate with HVPG values in patients with hepatic fibrosis and in patients with cirrhosis (Gressner et al. 1986). However, the prediction of severe portal hypertension or esophageal varices by laminin levels was poor with a positive predictive value of 85% and a negative predictive value of 43% (Mal et al. 1988; Kondo et al. 1995).

Serum hyaluronic acid concentrations also showed correlation with HVPG (Kropf et al. 1991), but as with laminin, its clinical application is limited because of low predictive value for the presence of severe PHT and EVs.

Another type of fibrosis marker was introduced, and it was called Fibrotest (Kropf et al. 1991). This marker is actually a panel of biochemical markers of hepatic fibrosis, and it combines the following five serum markers, all independently related to fibrosis as well as age and gender: alpha2-macroglobulin, haptoglobin, gamma glutamyl transferase (GGT), total bilirubin, and apolipoprotein A1. One study (Thabut et al. 2007) has shown that there is significant correlation between FibroTest values and HVPG values, but this correlation was weaker in patients with cirrhosis. Although the FibroTest value was significantly higher in patients with severe portal hypertension, the area under the receiver operating characteristic curve for the diagnosis of severe portal hypertension was only 0.79. Other studies are needed to evaluate the ability of Fibrotest to predict severe PHT, especially in patients with non-decompensated cirrhosis.

One study investigated the potential of other ECM proteins for detection of PHT (Leeming et al. 2013). The markers measured were C1M (type I-collagen), C3M and PRO-C3 (type III collagen), C4M and P4NP 7S (type IV collagen), C5M (type V collagen), C6M (type VI collagen), BGM (biglycan), ELM (elastin), CRPM (CRP). All ECM markers except for CRPM correlated significantly with HVPG. The combination of PRO-C3, C6M, and ELM provided better description of PHT, and a model combining the MELD Score with PRO-C3 and ELM provided odds ratios of >100 for having clinical significant PHT.

Inflammatory Biomarkers

The rationale for screening inflammatory serum biomarkers of portal hypertension is based on the fact that portal hypertension is pathogenically related to liver injury and fibrosis and that in turn these are associated with the activation of inflammatory pathways (Chojkier 1998; Picchiotti et al. 1994). One study found that the novel inflammatory biomarkers IL-1β, IL-1Rα, Fas-R, VCAM-1, TNFβ, and HSP-70 significantly correlated with HVPG in compensated cirrhosis (Buck et al. 2014). By using multivariate logistic regression analysis and the composite test of TGFβ; HSP-70; status of at risk of alcohol use; and Child class B, HVPG $>$ 12 mmHg could be exclude with 86% accuracy and with the sensitivity of 87.01%.

CD163 is a macrophage lineage-specific haemoglobin-haptoglobin scavenger receptor and a specific marker for macrophage activation (Schaer et al. 2005). The serum concentrations of the soluble form of CD163 (sCD163) are elevated during conditions of macrophage activation and proliferation. Elevated circulating sCD163 has been demonstrated in viral hepatitis, acute liver failure, and cirrhosis (Moller et al. 2007; Hiraoka et al. 2005; Holland-Fischer et al. 2011). One study have showed a positive correlations between sCD163 and HVPG, and the HVPG rose steeply to an asymptote of 22 mmHg with sCD163 up to about 5 mg/L and not to higher values with higher sCD163 (Grønbaek et al. 2012). sCD163 $>$ 3.95 mg/L (upper normal limit) predicted HVPG $\geq$10 mmHg with a positive predictive value of 0.99.

Metabolic Parameters

Obesity and metabolic abnormalities have been identified as an independent predictor of clinical decompensation in patients with compensated cirrhosis of various etiologies (Berzigotti et al. 2011). One study investigated the relationship between metabolic variables, especially insulin resistance (IR) and adipocytokines, and portal hypertension in patients with cirrhosis (Eslam et al. 2013). The IR was measured by the homeostatic model for the assessment of IR (HOMA-IR), which is calculated by multiplying fasting plasma insulin by fasting plasma glucose, then dividing by the constant 22.5. The study showed that insulin resistance (IR) and serum levels of adiponectin, an adipocyte-derived hormone, significantly correlated with HVPG, and both parameters independently predict the presence of esophageal varices, with an odds ratio of 2.01 for high HOMA-IR score and 1.97 for high adiponectin levels. The HOMA-IR also predicted variceal bleeding, patients with HOMA < 4 had a significantly higher probability of having free survival of risk of variceal bleeding than patient with HOMA > 4 (97% vs 67..4%. $P = 0.001$).

Potential Applications of Serum Biomarkers of Portal Hypertension to Prognosis, Other Diseases or Conditions

For many decades, studies have been done to identify serum biomarkers that can predict the existence of portal hypertension in patients with chronic liver disease. The measurement of the HVPG is the gold-standard technique for the evaluation of PHT in liver disease. In patients with cirrhosis, HVPG measurements provide independent prognostic information on survival and the risk of decompensation and complications. Variceal bleeding is the most feared complication of portal hypertension. The appearance of varices in patients with compensated cirrhosis is associated with an increased risk of death (1.0%–3.4% per year), and the occurrence of variceal bleed significantly increases this risk, with 1-year mortality rate as high as 57%. Approximately 20% of deaths occur in the first 6 weeks of a bleeding episode (D'Amico et al. 2006). It is of most importance to do bleeding risk stratification in patients with cirrhosis. Risk factors for variceal bleeding include morphologic characteristics of varices viewed via esophagogastroduodenoscopy (large varices, red wale markings), cirrhosis severity determined by Child-Turcotte-Pugh scoring (Class B or C), and elevated HVPG. Primary prophylaxis of esophageal varices is recommended for patients at high risk for bleeding. The prophylactic options include pharmacologic agents, especially nonselective b-adrenergic blockers, such as propranolol and nadolol, and the endoscopic prophylactic intervention variceal band ligation, and both options are effective in reducing the risk of bleeding. B-blockers have been shown to significantly reduce portal pressure, as measured by HVPG, significantly reduce the risk of a first bleeding episode, and significantly reduce mortality (D'Amico et al. 1999). HVPG has been used to evaluate the hemodynamic response to b-blockers. An HVPG reduction to less than 12 mmHg essentially eliminates the risk of bleeding and improves survival (Groszmann et al. 1990).

It is recommended to perform screening endoscopy at 2–3 years interval in patients without varices and at 1–2 years interval in patients with small varices to evaluate the development and/or progression of varices (DeFranchis 2000). It was estimated that 100 screening endoscopy needs to be performed to prevent 1–2 cases of variceal bleeding (Boyer 1997).

Identification of biomarkers of portal hypertension and esophageal varices will allow upper gastrointestinal tract endoscopy to be carried out only in selected group of patients thus avoid unnecessary intervention and at the same time not to miss patients at risk of bleeding (Sarwar et al. 2004). Any surrogate biomarker of HVPG is a future candidate to be a method used for monitoring response to pharmacologic prophylaxis, either primary or secondary, without doing invasive procedures such as endoscopy and catheterization of the hepatic vein.

Summary

- Portal hypertension is responsible for the fatal complications of cirrhosis such as variceal bleeding.
- The most accurate method for evaluating the presence and severity of portal hypertension is the measurement of the hepatic venous pressure gradient.
- It is invasive, costly, and not available in all centers. Several alternative noninvasive techniques have been proposed to assess portal hypertension, including serum molecules (biomarkers) and imaging techniques.
- This chapter focused on the potential role of multiple serum markers of portal hypertension that could be clinically applied to predict the presence of clinically significant portal hypertension, to stratify patients with respect to the severity of portal hypertension, to predict lethal complications such as variceal bleeding, and to monitor disease progression or treatment response without exposing patients to the risks of repeated invasive assessment.

References

Baranova A, Lal P, Birerdinc A, Younossi ZM. Non-invasive markers for hepatic fibrosis. BMC Gastroenterol. 2011;11:91.

Bataller R, Brenner DA. Liver fibrosis. J Clin Invest. 2005;115:209–18.

Berzigotti A, Gilabert R, et al. Noninvasive prediction of clinically significant portal hypertension and esophageal varices in patients with compensated liver cirrhosis. Am J Gastroenterol. 2008;103(5):1159–67.

Berzigotti A, Garcia-Tsao G, et al. Obesity is an independent risk factor for clinical decompensation in patients with cirrhosis. Hepatology. 2011;54(2):555–61.

Bosch J, Abraldes JG, Berzigotti A, García-Pagan JC. The clinical use of HVPG measurements in chronic liver disease. Nat Rev Gastroenterol Hepatol. 2009;6:573–82.

Boyer T. Natural history of portal hypertension. Clin liver dis. 1997;1:31–44.

Braillon A, Cales P, et al. Influence of the degree of liver failure on systemic and splanchnic haemodynamics and on response to propranolol in patients with cirrhosis. Gut. 1986;27(10):1204–9.

Buck M, Garcia-Tsao G, et al. Novel inflammatory biomarkers of portal pressure in compensated cirrhosis patients. Hepatology. 2014;59(3):1052–9.
Bureau C, Péron JM, Alric L, Morales J, Sanchez J, Barange K, et al. A la carte treatment of portal hypertension: adapting medical therapy to hemodynamic response for the prevention of bleeding. Hepatology. 2002;36:1361–6.
Chalasani N, Kahi C, Francois F, Pinto A, Marathe A, Bini EJ, Pandya P, Sitaraman S, Shen J. Improved patient survival after acute variceal bleeding: a multicenter, cohort study. Am J Gastroenterol. 2003;98:653–9.
Chojkier M. Regulation of collagen gene expression. In: Strain A, Diehl A, editors. Liver growth and repair. London: Chapman & Hall; 1998. p. 430–50.
D'Amico G, Pagliaro L, Bosch J. Pharmacological treatment of portal hypertension: an evidence-based approach. Semin Liver Dis. 1999;19(4):475–505.
D'Amico G, Garcia-Tsao G, Pagliaro L. Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. J Hepatol. 2006;44(1):217–31.
DeFranchis R. Updating consensus in portal hypertension: report of the Baveno III consensus workshop on definitions, methodology and therapeutic strategies in portal hypertension. J Hepatol. 2000;33:846–52.
Eslam M, Ampuero J, et al. Predicting portal hypertension and variceal bleeding using non-invasive measurements of metabolic variables. Ann Hepatol. 2013;12(4):588–98.
Ferlitsch M, Reiberger T, et al. von Willebrand factor as new noninvasive predictor of portal hypertension, decompensation and mortality in patients with liver cirrhosis. Hepatology. 2012;56(4):1439–47.
Feu F, García-Pagán JC, Bosch J, Luca A, Terés J, Escorsell A, et al. Relation between portal pressure response to pharmacotherapy and risk of recurrent variceal haemorrhage in patients with cirrhosis. Lancet. 1995;346:1056–9.
Forestier J, Dumortier J, Guillaud O, et al. Noninvasive diagnosis and prognosis of liver cirrhosis: a comparison of biological scores, elastometry, and metabolic liver function tests. Eur J Gastroenterol Hepatol. 2010;22:532–40.
Garcia-Tsao G, Sanyal AJ, et al. Prevention and management of gastroesophageal varices and variceal hemorrhage in cirrhosis. Am J Gastroenterol. 2007;102(9):2086–102.
Giannini EG. Review article: thrombocytopenia in chronic liver disease and pharmacologic treatment options. Aliment Pharmacol Ther. 2006;23(8):1055–65.
Gluud C, Henriksen J, Nielsen G, et al. Prognostic indicators in alcoholic cirrhotic men. Hepatology. 1988;8(2):222–7.
Gordon A, McLean CA, et al. Hepatic steatosis in chronic hepatitis B and C: predictors, distribution and effect on fibrosis. J Hepatol. 2005;43(1):38–44.
Gressner AM, Weiskirchen R. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. J Cell Mol Med. 2006;10:76–99.
Gressner AM, Tittor W, Negwer A, Pick-Kober KH. Serum concentration of laminin and aminoterminal propeptide of type III procollagen in relation to the portal venous pressure of fibrotic liver diseases. Clin Chim Acta. 1986;161:249–58.
Grønbaek H, Sandahl TD, et al. Soluble CD163, a marker of Kupffer cell activation, is related to portal hypertension in patients with liver cirrhosis. Aliment Pharmacol Ther. 2012;36(2): 173–80.
Groszmann RJ, Bosch J, Grace ND, et al. Hemodynamic events in a prospective randomized trial of propranolol versus placebo in the prevention of a first variceal hemorrhage. Gastroenterology. 1990;99(5):1401–7.
Hametner S, Ferlitsch A, et al. The VITRO Score (Von Willebrand Factor Antigen/Thrombocyte Ratio) as a new marker for clinically significant portal hypertension in comparison to other nonInvasive parameters of fibrosis including ELF test. PLoS One. 2016;11(2):e0149230.
Hiraoka A, Horiike N, Akbar SM, Michitaka K, Matsuyama T, Onji M. Expression of CD163 in the liver of patients with viral hepatitis. Pathol Res Pract. 2005;201:379–84.

Hoefs JC. Serum protein concentration and portal pressure determine the ascitic fluid protein concentration in patients with chronic liver disease. J Lab Clin Med. 1983;102:260–73.
Holland-Fischer P, Gronbaek H, Sandahl TD, et al. Kupffer cells are activated in cirrhotic portal hypertension and not normalised by TIPS. Gut. 2011;60:1389–93.
Iwakiri Y, Groszmann RJ. Vascular endothelial dysfunction in cirrhosis. J Hepatol. 2007;46(5): 927–34.
Iwakiri Y, Grisham M, Shah V. Vascular biology and pathobiology of the liver: report of a single-topic symposium. Hepatology. 2008;47:1754–63.
Kondo M, Miszputen SJ, Leite-Mor MM, Parise ER. The predictive value of serum laminin for the risk of variceal bleeding related to portal pressure levels. Hepatogastroenterology. 1995;42:542–5.
Kropf J, Gressner AM, Tittor W. Logistic-regression model for assessing portal hypertension by measuring hyaluronic acid (hyaluronan) and laminin in serum. Clin Chem. 1991;37:30–5.
La Mura V, Reverter JC, Flores-Arroyo A, Raffa S, Reverter E, Seijo S, et al. Von Willebrand factor levels predict clinical outcome in patients with cirrhosis and portal hypertension. Gut. 2011; 60(8):1133–8.
Lavi S, Yang EH, Prasad A, et al. The interaction between coronary endothelial dysfunction, local oxidative stress, and endogenous nitric oxide in humans. Hypertension. 2008;51:127–33.
Lebrec D, Sogni P, Vilgrain V. Evaluation of patients with portal hypertension. Clin Gastroenterol. 1997;11:221–41.
Leeming DJ, Karsdal MA, et al. Novel serological neo-epitope markers of extracellular matrix proteins for the detection of portal hypertension. Aliment Pharmacol Ther. 2013;38(9):1086–96.
Lim YL, Choi E, et al. Clinical implications of the serum apelin level on portal hypertension and prognosis of liver cirrhosis. Gut Liver. 2016;10(1):109–16.
Lisman T, Leebeek FW, de Groot PG. Haemostatic abnormalities in patients with liver disease. J Hepatol. 2002;37:280–7.
Lluch P, Torondel B, Medina P, et al. Plasma concentrations of nitric oxide and asymmetric dimethylarginine in human alcoholic cirrhosis. J Hepatol. 2004;41(1):55–9.
Mal F, Hartmann DJ, Trinchet JC, Lacombe F, Ville G, Beaugrand M. Serum laminin and portal pressure in alcoholic cirrhosis. A study of 39 patients. Gastroenterol Clin Biol. 1988;12:841–4.
Merkel C, Montagnese S. Should we routinely measure portal pressure in patients with cirrhosis, using hepatic venous pressure gradient (HVPG) as guidance for prophylaxis and treatment of bleeding and re-bleeding?Yes! Eur J Intern Med. 2011;22:1–4.
Merkel C, Bolognesi M, Sacerdoti D, Bombonato G, Bellini B, Bighin R, et al. The hemodynamic response to medical treatment of portal hypertension as a predictor of clinical effectiveness in the primary prophylaxis of variceal bleeding in cirrhosis. Hepatology. 2000;32:930–4.
Moitinho E, Escorsell A, Bandi JC, Salmerón JM, García-Pagán JC, Rodés J, Bosch J. Prognostic value of early measurements of portal pressure in acute variceal bleeding. Gastroenterology. 1999;117:626–31.
Moller HJ, Gronbaek H, Schiodt FV, et al. Soluble CD163 from activated macrophages predicts mortality in acute liver failure. J Hepatol. 2007;47:671–6.
Mookerjee RP, Mehta G, et al. Hepatic dimethylarginine-dimethylaminohydrolase1 is reduced in cirrhosis and is a target for therapy in portal hypertension. J Hepatol. 2015;62(2):325–31.
Picchiotti R, Mingazzini PL, Scucchi L, Bressan M, Di Stefano D, Donnetti M, et al. Correlations between sinusoidal pressure and liver morphology in cirrhosis. J Hepatol. 1994;20:364–9.
Ripoll C, Bañares R, Rincón D, Catalina MV, Lo Iacono O, Salcedo M, Clemente G, Núñez O, Matilla A, Molinero LM. Influence of hepatic venous pressure gradient on the prediction of survival of patients with cirrhosis in the MELD era. Hepatology. 2005;42:793–801.
Sanyal AJ, Genning C, Reddy KR, Wong F, Kowdley KV, Benner K, et al. The North American study for the treatment of refractory ascites. Gastroenterology. 2003;124:634–41.
Sarwar S, Khan AA, et al. Non-endoscopic prediction of esophageal varices in cirrhosis. J Coll Physicians Surg Pak. 2004;15(9):528–31.

Schaer DJ, Schleiffenbaum B, Kurrer M, et al. Soluble hemoglobin-haptoglobin scavenger receptor CD163 as a lineagespecific marker in the reactive hemophagocytic syndrome. Eur J Haematol. 2005;74:6–10.

Schuppan D, Ruehl M, Somasundaram R, Hahn EG. Matrix as a modulator of hepatic fibrogenesis. Semin Liver Dis. 2001;21:351–72.

Shin WG, Park SH, Jun S-Y, et al. Simple tests to predict hepatic fibrosis in nonalcoholic chronic liver diseases. Gut Liver. 2007;1:145–50.

Shin WG, Park SH, Jang MK, et al. Aspartate aminotransferase to platelet ratio index (APRI) can predict liver fibrosis in chronic hepatitis B. Dig Liver Dis. 2008;40:267–74.

Stanley AJ, Robinson I, et al. Haemodynamic parameters predicting variceal haemorrhage and survival in alcoholic cirrhosis. QJM. 1998;91(1):19–25.

Thabut D, Imbert-Bismut F, et al. Relationship between the Fibrotest and portal hypertension in patients with liver disease. Aliment Pharmacol Ther. 2007;26(3):359–68.

Van Mourik JA, Boertjes R, Huisveld IA, et al. von Willebrand factor propeptide in vascular disorders: a tool to distinguish between acute and chronic endothelial cell perturbation. Blood. 1999;94:179–85.

Vizzutti F, Romanelli RG, Arena U, et al. ADMA correlates with portal pressure in patients with compensated cirrhosis. Eur J Clin Invest. 2007;37(6):509–15.

Wai C-T, Greenson JK, Fontana RJ, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology. 2003;38:518–26.

Wai CT, Cheng CL, Wee A, et al. Non-invasive models for predicting histology in patients with chronic hepatitis B. Liver Int. 2006;26:666–72.

Weiler-Normann C, Herkel J, Lohse AW. Mouse models of liver fibrosis. Z Gastroenterol. 2007;45:43–50.

Zaman A, Hapke R, Flora K, Rosen HR, Benner K. Factors predicting the presence of esophageal or gastric varices in patients with advanced liver disease. Am J Gastroenterol. 1999;94(11): 3292–6.

Biomarkers for Recurrence of Hepatocellular Carcinoma

8

Seow Chong Lee, Hwee Tong Tan, and Maxey Ching Ming Chung

Contents

S.C. Lee • H.T. Tan • M.C.M. Chung (✉)
Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore
e-mail: a0024010@u.nus.edu; bchtht@nus.edu.sg; maxey_chung@nuhs.edu.sg

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_9

Abstract

Hepatocellular carcinoma (HCC) is one of the deadliest cancers in the world, and the prognosis of HCC patients remains poor despite earlier diagnosis and treatment. Recurrence is the main cause of death in patients who received curative treatment for the primary cancer. Tumors arising within 2 years of treatment typically originate from the primary tumour. These recurrent tumours tend to be aggressive and result in high mortality rate for these patients. Hence there is a clinical need to develop prognostic biomarkers for prediction of early recurrence. Proteomics involves the unbiased study of global changes of protein expression in a high throughput manner. This allows for identification of biomarkers for prediction of HCC recurrence. In this review, we described the proteomics workflow and platforms used in the biomarker discovery for HCC recurrence. We also summarized the main findings of studies that utilized proteomics as the primary tool for biomarker identification in patient samples. Lastly, we discussed the possible improvements in study design and technologies to translate the results obtained from proteomic studies to clinical use.

Keywords

Hepatocellular carcinoma • Intrahepatic metastasis • Mass spectrometry • Proteomics • Recurrence

List of Abbreviations

2D-DIGE	2-Dimensional-difference gel electrophoresis
2-DE	2-Dimensional electrophoresis
adoMET	S-Adenosylmethionine
AMACR	Alpha-methylacyl-CoA racemase
cICAT	Cleavable isotope-coded affinity tags
emPAI	Exponentially modified protein abundance index
HCC	Hepatocellular carcinoma
IEF	Isoelectric focusing
IHC	Immunohistochemistry
iTRAQ	Isobaric tags for relative and absolute quantitation
LC	Liquid chromatography
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
NDRG1	N-myc downstream-regulated gene 1
PAGE	Polyacrylamide gel electrophoresis
p*I*	Isoelectric point
qRT-PCR	Quantitative real-time-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SELDI	Surface-enhanced laser desorption/ionization

TMA	Tissue microarray analysis
TOF	Time of flight

Key Facts of Hepatocellular Carcinoma Recurrence

- Up to 80% of all hepatocellular carcinoma patients who received curative treatment suffer from recurrence.
- Recurrence is broadly divided into two types: intrahepatic metastasis and multicentric occurrence.
- Intrahepatic metastasis is associated with early recurrence (within 2 years after treatment), poor overall survival, and aggressive metastatic nature of recurrent tumors.
- Clonal analysis suggests that most of the tumors in early recurrence originate from the primary tumor.
- There are no effective prophylactic treatments available to prevent recurrence.
- Proteomic analyses of primary tumors in early recurrent patients aim to identify biomarkers for prediction and prophylactic treatment of early recurrence.

Definitions of Words and Terms

2-Dimensional electrophoresis	This is a proteomic technique involving the separation of proteins based on two different properties. Typically, proteins are first separated by their isoelectric point, followed by separation according to molecular weight.
Biomarker	A biomarker is a biological molecule that is characteristic of disease states or processes in the body. The levels of biomarkers can be detected and measured in the body.
Hepatocellular carcinoma	Hepatocellular carcinoma is the most common form of liver cancer. This form of cancer arises from neoplastic changes to the hepatocytes.
Intrahepatic metastasis	Intrahepatic metastasis is the formation of recurrent tumors that originate from the primary tumor. This phenomenon is strongly associated with venous invasion of the primary tumor to the portal vein.
Liquid chromatography	Liquid chromatography separates analytes in a complex mixture based on the strength of interaction between the stationary phase in the

	analytical column and the analytes in the liquid mobile phase.
Mass spectrometry	Mass spectrometry is an analytical method that detects the analyte ion in the gaseous phase which is specific to the mass over charge ratio of the analyte. In proteomics, peptides are first ionized and then converted to gaseous phase ions. The identity of the peptide can be determined by comparing the mass spectrum generated by tandem mass spectrometry analysis with a reference database. Protein identification is inferred by detecting tryptic peptides that are unique for each protein.
Multicentric carcinogenesis	Multicentric carcinogenesis refers to the formation of recurrent tumors that have different clonal origins as the primary tumors. These tumors can be considered as new primary tumors, and they are believed to be formed due to the accumulation of genetic changes in the cirrhotic liver.
Proteomics	Proteomics is the large-scale study of proteins to characterize biological processes. Expression proteomics is commonly used in biomarker discovery studies, in which quantitative/semiquantitative information of protein expression levels are obtained and compared between different disease states.
Sensitivity	Sensitivity refers to the ability to correctly identify true positives in the population. In a diagnostic test for a disease, the test with high sensitivity would be able to identify most of the patients with the disease.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis	This involves the separation of proteins by their molecular weight in denaturing conditions. The proteins in the samples are first denatured and reduced by addition of sodium dodecyl sulfate, reducing agents and heating. Proteins in the samples are separated in the polyacrylamide gel under the influence of an electric field.
Specificity	Specificity refers to the ability to correctly identify true negatives in the population. In a diagnostic test for a disease, the test with high specificity would be able to correctly identify patients without the disease and minimize placing patients without disease into the disease group (false positives).

Introduction

HCC is the fifth most prevalent cancer in the world, with increasing incidence in both Western and Asian countries (Ferlay et al. 2015). Technological advances in imaging have enabled the early detection and diagnosis of HCC (Mancuso 2013). However, the prognosis of HCC patients remains poor. HCC is the third most frequent cause of cancer-related death, with up to 80% of patients suffering from recurrence after treatment with curative intent such as liver transplantation, resection, or ablation (Franssen et al. 2014; Lau and Lai 2008; Poon et al. 2000). Recurrence is the main cause of cancer-related death in patients amenable to curative treatment. Hence, there is the clinical need to identify patients with higher risk of recurrence so that they could benefit from increased surveillance. These proteins might also be potential drug targets for development of novel prophylactic treatment to prevent recurrence.

HCC recurrence can arise from the undetected microscopic spread of the primary tumor (intrahepatic metastasis) or de novo formation of new tumors on the cirrhotic liver which provides a favorable "field" for accumulation of genetic alterations, eventually leading to neoplasia (multicentric carcinogenesis) (Sherman 2008). The risk factors and outcomes for these two types of recurrence are vastly different (Cheng et al. 2015; Du et al. 2014; Portolani et al. 2006; Wu et al. 2009). Intrahepatic metastasis usually occurs within 2 years of initial treatment. The early recurrent tumors are usually aggressive and refractory to subsequent treatment, resulting in a lower 5-year survival rate compared to patients with late recurrence. Risk factors for early recurrence are tumor related, of which venous invasion is the most common factor. Clonality analysis demonstrated that the recurrent tumors originate from the primary HCC (Morimoto et al. 2003; Wang et al. 2013; Zhang et al. 2015). Multicentric carcinogenesis occurs more than 2 years after treatment (late recurrence). The recurrent tumors are of different clonal origin; hence, they can be considered as new primary tumors. Most patients with multicentric carcinogenesis are suitable for treatment, and these patients have a comparable survival rate to that of the cirrhotic patients with a higher risk of developing primary HCC (Cucchetti et al. 2009). The risk factors for late recurrence are related to host factors such as degree of cirrhosis and function of remnant liver.

The poor prognosis of early recurrence and its correlation with the primary HCC led to studies on the primary tumor to decipher the molecular basis of recurrence and determine biomarkers that can predict the risk of early recurrence. Proteomics provides a platform for large-scale studies of the proteome, in which differentially expressed proteins can be used as biomarkers to segregate patients according to risk of early recurrence. Furthermore, the expression patterns of the proteins allow for identification of molecular pathways that are dysregulated in the tumors of early recurrence patients, thus allowing for development of novel therapeutics that target key players involved in activation/inactivation of the pathways. In this review, we will outline the typical proteomic workflow in identification of potential prognostic biomarkers for HCC recurrence. Different techniques used in the separation and

quantitation of proteins/peptides will be discussed. In addition, we will summarize the findings from studies that utilized proteomics in the identification of biomarkers for early recurrence. Lastly, we will discuss the improvements that can be made to translate results generated from proteomics to clinical use.

Proteomics as a Tool for Discovery of Cancer Biomarkers

The typical workflow in proteomic identification of biomarkers is illustrated in Fig. 1. Most studies used liver tissues from the tumor and the adjacent non-tumor regions for proteomic-based discovery studies. Other possible samples include biological fluids such as serum/plasma, as they may contain proteins that are shred or actively secreted by the tumors into the blood circulation. These samples are then processed prior to separation, quantitation, and identification of proteins or peptides by proteomics. Proteomic technologies used in the separation and quantitation can be broadly divided into gel-based, liquid chromatography (LC)-based, and chip-based methods. Identification of proteins is usually achieved by tandem mass spectrometry (MS/MS) analysis of tryptic peptides. Finally, validation is performed to confirm the differential expression of proteins identified in the discovery phase in a separate cohort of clinical samples.

Validation is usually performed using antibody-based methods, such as Western blotting, immunohistochemistry (IHC), and tissue microarray analysis (TMA). Results from these assays would be analyzed in conjunction with clinical information of the patients to identify correlations of protein expression with prognosis of HCC. Functional studies can be performed on cell lines or animal models to correlate the changes in protein expression levels with different traits leading to cancer recurrence, thus providing an understanding of the biological pathways involved in HCC recurrence. After the clinical relevance of target protein has been established, the focus would be shifted to the development of a detection platform that allows for sensitive and reproducible analysis of target protein expression in the clinic. The following sections describe the different approaches that were applied in published studies that involved identification of HCC recurrence biomarkers, with emphasis on the proteins' separation and quantitation.

Gel-Based Proteomic Approaches

Gel-based proteomics involves the separation of proteins present in a complex sample by the polyacrylamide gel. 2-Dimensional electrophoresis (2-DE) is the main workhorse of gel-based proteomics, in which proteins are first separated by their isoelectric point (p*I*) in isoelectric focusing (IEF), followed by molecular size via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels are subsequently stained to visualize the protein spots. Relative quantitation of

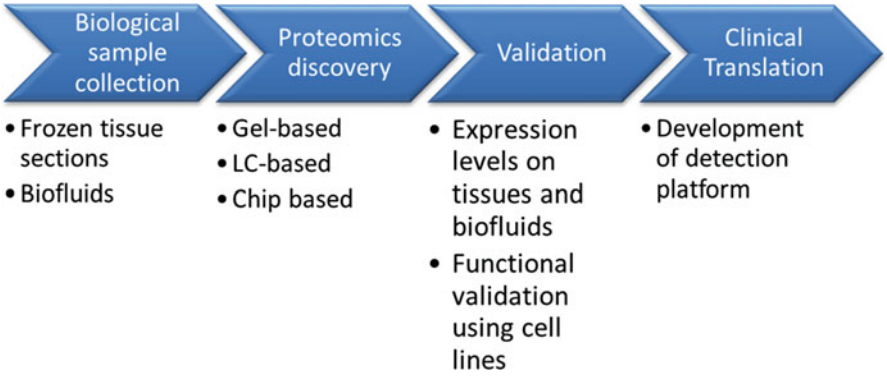

Fig. 1 Proteomic workflow for biomarker discovery. The typical proteomic workflow involves collection of biological samples that are relevant to the research question. Samples are then processed for application on the proteomic platform. Validation of protein expression and function is carried out to confirm the findings observed in the proteomic study and to provide evidence to the functional relevance of protein dysregulation in the disease of interest. After clinical validation, the focus will be shifted to the development of a suitable platform that would allow for detection of target proteins with high accuracy and reproducibility in the clinical setting

protein spots is achieved by comparing the intensity of protein spots with matched p*I* and molecular size in different gels.

Conventional 2-DE suffers from lack of reproducibility of the spot patterns and intensities due to the high levels of inherent technical and systematic variability (Voss and Haberl 2000). This results in the difficulty to distinguish true biological variations from technical artifacts. 2-Dimensional-difference gel electrophoresis (2D-DIGE) was introduced by Minden's lab to minimize technical variations associated with conventional 2-DE (Unlu et al. 1997). This decrease in variation is brought about by the simultaneous separation of two different samples on a single 2-DE experiment. In addition, an additional sample can be labeled by Cy2, which can serve as an internal standard to remove technical variations arising from individual 2-DE experiments during data analysis. The workflow for 2D-DIGE is illustrated in Fig. 2. Readers are recommended to refer to a recent review (Arentz et al. 2015) for a comprehensive understanding of the technical considerations in the design of 2D-DIGE experiments.

Liquid Chromatography-Based Proteomic Approaches

LC-based proteomics involves the tryptic digestion of proteins into peptides, before separation of peptides by LC to reduce the complexity of the samples. The mode of separation depends on the chemistry of the column, among which strong cation exchange and reverse phase columns are commonly used. These columns separate

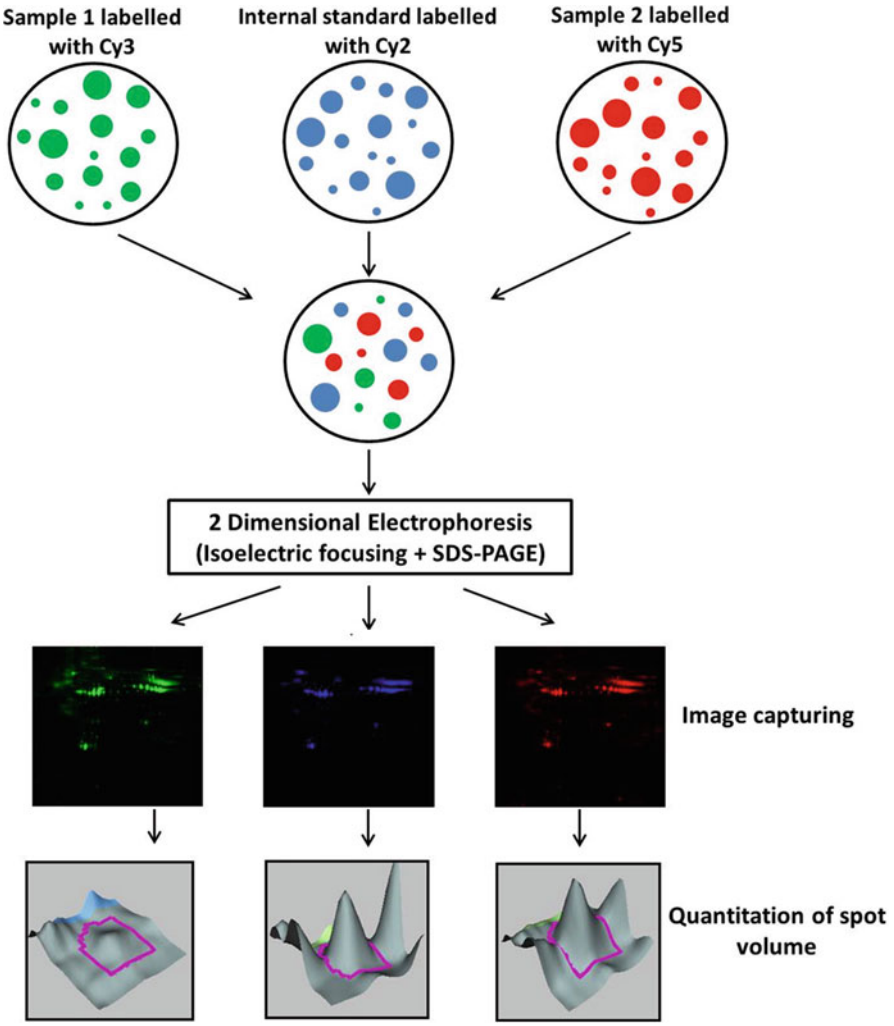

Fig. 2 2D-DIGE workflow. In 2D-DIGE, two different protein samples are labeled with Cy3 and Cy5, respectively. An internal reference sample, which is usually formed by pooling equal amounts of all samples used in the DIGE experiment, is labeled with Cy2. These three samples are then mixed, before the proteins are subjected to separation by isoelectric focusing and gel electrophoresis. The protein maps of different samples are visualized and captured by excitation of the fluorophores on the CyDyes. The images are exported to software for processing and normalization procedures. Relative quantitation is obtained by comparing the volumes of matched protein spots in different samples

the peptides based on charge and hydrophobicity, respectively. Identification of proteins in the sample is achieved by MS/MS analysis of the peptides. Various methods of protein quantitation are available, including labeling (chemical labeling) and label-free methods (spectrum counting), which will be discussed in the following sections.

Chemical Labeling: Cleavable Isotope-Coded Affinity Tags (cICAT) and Isobaric Tags for Relative and Absolute Quantitation (iTRAQ)

The proteins in the samples can be labeled with chemical tags for quantitation. The labeling process involves the formation of a chemical bond between the tag and the protein/peptide, followed by mixing of labeled samples before LC separation and MS analysis. Examples of chemical labeling tags include cICAT (Gygi et al. 1999) and iTRAQ (Choe et al. 2007; Ross et al. 2004). cICAT and iTRAQ differ in the labeling and method of obtaining quantitative data from mass spectrometry. cICAT labels carry a sulfhydryl reactive group, linker region containing either light or heavy isotopes ($^{12}C/^{13}C$), and a biotin moiety. Protein samples are labeled and trypsinized, and the labeled peptides are purified by avidin affinity chromatography. Cleavage of biotin tag is required as the full tag is too big and interferes with ionization of the peptides. Peaks corresponding to the same peptide from the two different samples will appear as a doublet with a mass difference of 9 Da. Quantitative information can be obtained by comparing the peak intensity of the two distinct peaks in the MS spectrum.

In contrast, the proteins from the different samples are trypsinized before labeling with the iTRAQ labels. These labels contain an amine-reactive group, a balance group, and a reporter mass (114 to 117 for 4-plex, 113–121, excluding 120, for 8-plex). The different labels are isobaric and thus have the same mass and chemical properties. Samples are mixed after labeling and subsequently subjected to LC-MS analysis. The precursor ions from all the different samples will elute at the same time for MS analysis. Quantitative information is obtained in the MS/MS analysis, in which the reporter ions will be dissociated and their peak intensities can be compared for determination of relative abundance of proteins. Both cICAT and iTRAQ workflows involve mixing of labeled samples before LC-MS analysis, thus allowing for multiplexing of samples and avoiding technical variations arising from LC separation and MS analysis. The workflows for cICAT and iTRAQ are summarized in Fig. 3.

Label-Free Proteomic Approach

In a label-free proteomic approach, different samples are processed separately during LC-MS analysis. Spectrum counting is the most basic label-free quantitation method, which is based on the fact that an increased abundance of proteins will result in an increased number and frequency of peptides observed (Washburn et al. 2001). Spectrum counting methods can be used to determine the relative abundance of the protein by using exponentially modified protein abundance index (emPAI) (Ishihama et al. 2005). Recently, a novel MS strategy, termed SWATH-MS (Gillet et al. 2012), has been developed. SWATH-MS utilizes a sequential mass window to acquire MS/MS information of all detectable peptides within that mass window. The information acquired can be used to generate a complete fragment ion map of the proteome. Quantitative information can be obtained from the extracted

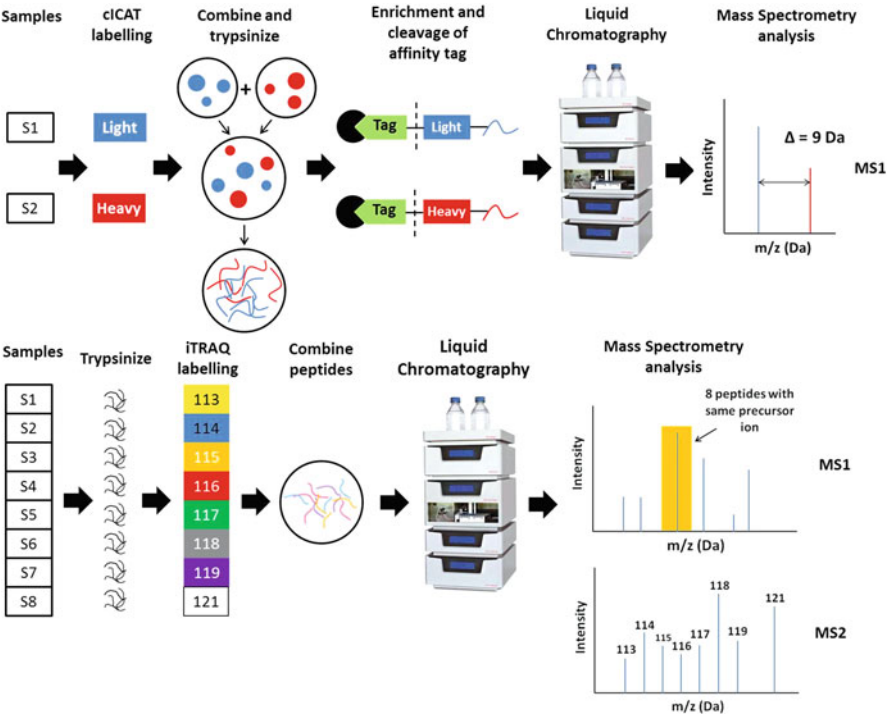

Fig. 3 Workflow of (**a**) cICAT and (**b**) iTRAQ analysis. Pictorial representation of the workflow of (**a**) cICAT and (**b**) iTRAQ analysis. (**a**) cICAT analysis involves the labeling of proteins of two different samples with cICAT labels containing either light or heavy isotopes of carbon. The two samples are then mixed, trypsin digested before enrichment of tagged peptides. The biotin tags on the labeled peptides are then cleaved before the peptides are subjected to liquid chromatography-mass spectrometry analysis. Peptides of the same protein will appear as a doublet with a mass difference of 9 Da in mass spectrometry analysis (MS1). (**b**) iTRAQ analysis involves trypsinization of individual samples, before labeling of up to eight different samples with the iTRAQ reagents. The labeled peptides are combined, before subjected to liquid chromatography-tandem mass spectrometry analysis. Quantitative information is obtained by comparing the relative intensity of the reporter ions after tandem mass spectrometry analysis (MS2)

ion chromatogram of the peptides. SWATH-MS strategy allows for digital archiving of tumor proteome, allowing retrospective quantitative comparison of targeted proteins in different samples when new research questions arise.

Chip-Based Proteomics: Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS)

SELDI-TOF-MS combines retentate chromatography and mass spectrometry on a chip to identify biomarkers for analytical purposes (Issaq et al. 2002). These chips contain ligands that have been derivatized with different chromatographic

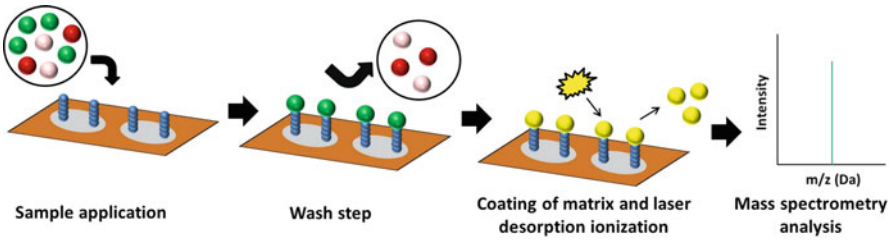

Fig. 4 SELDI-TOF-MS workflow. Samples are applied to ProteinChips, which are derivatized with chromatography ligands. Some of the chromatographic chemistries available include ion exchange, reverse phase, normal phase, and immobilized metal affinity chromatography. The unbound proteins are then washed away. Subsequently, the proteins are coated with a layer of matrix, which is necessary for laser desorption ionization. Quantitative information is obtained by comparing intensities of the peaks with the same mass-to-charge ratios in different samples

chemistries. The procedure involves sample application, washing of unbound proteins, coating of matrix, and analysis of proteins present on chip by MS. Quantitative information is obtained by comparing the area under peaks with defined *m*/*z* values. One drawback of SELDI-TOF-MS is that the identity of the peptide that produces the particular peak with the *m*/*z* value is unknown, and further analysis is required to identify the protein of interest. The SELDI-TOF-MS workflow is summarized in Fig. 4.

Biomarkers of HCC Recurrence Identified from Proteomic Studies

For the selection of publications to be included in this review, the PubMed database was searched with the term "((Hepatocellular carcinoma) AND Recurrence) AND Proteomics)." We selected papers that utilized biological samples obtained from patients and the usage of proteomics as the primary tool for biomarker discovery. There are a total of ten publications that fulfilled this requirement. Nine of the publications utilized frozen tissue samples, while only one publication used serum for biomarker discovery. The details of the publications are summarized in Table 1. Most of the publications focused on the discovery of candidate biomarkers based on their discriminatory powers between patients with or without cancer recurrence. The other publications are based on their discovery or validation criteria of known tumor factors and pathways associated with poor prognosis of HCC. These publications would be discussed in greater detail in the subsequent sections.

Biomarker Discovery Based on Time to Recurrence

The first group of studies grouped the patients solely on the presence and absence of recurrence within a stipulated time period for the discovery studies. These studies choose protein(s) with high discriminatory power in the discovery set for subsequent

Table 1 Proteomic studies for identification of HCC recurrence biomarkers. This table summarizes the main findings of the proteomic studies that attempted to identify predictive biomarkers for HCC recurrence. The papers are ordered in chronological order. The regulations of the candidate proteins are denoted by the *arrows*, where ↑ indicates an upregulation and ↓ indicates a downregulation of protein expression in recurrence patients compared to non-recurrence

Reference	Sample type	Curative treatment	Classification	Etiology	Discovery platform	Candidate biomarkers (regulation)	Validation
Yokoo et al. (2007)	Tumor tissues	Surgical resection	Recurrence within 6 months post surgery versus no recurrence 2 years post surgery	Mixed	2D-DIGE, MALDI-TOF/MS	Panel of 23 proteins (10 ↑ 13↓)	2D-DIGE
Orimo et al. (2008)	Tumor and non-tumor tissues	NA	Tissue differentiation	Mixed, mostly HCV	2D-DIGE, LC-MS/MS	Microtubule-associated protein RP/EB family member 1 (↑)	IHC
Yi et al. (2008)	Tumor, non-tumor, and normal liver tissues	Surgical resection	Recurrence versus no recurrence within 1 year post surgery	HBV	2-DE, MALDI-TOF/MS	Stress-70 protein, mitochondrial (↑)	IHC
Bai et al. (2009)	Tumor and non-tumor tissues	Liver transplantation	Recurrence versus no recurrence within 3 years post transplantation	HBV	cICAT, 2DLC-MS/MS	Calpain small subunit 1 (↓)	qRT-PCR, Western blot, IHC
Cheng et al. (2011)	Tumor tissues	Liver transplantation	Recurrence versus no recurrence (time frame not stated)	Not stated	2-DE, MALDI-TOF/MS	Protein NDRG1 (↑)	Western blot, IHC
Kanamori et al. (2011)	Tumor and non-tumor tissues	NA	Tumor versus adjacent non-tumor	HCV	2DLC-MS/MS	Talin-1 (↑)	IHC

Cao et al. 2013	Serum	Microwave ablation	Recurrence versus no recurrence within 1 year post curative MWA	HBV	SELDI-TOF-MS	m/z of 7,787, 6,858 and 6,646 (↓)	SELDI-TOF-MS
Tan et al. (2014)	Paired tumor and non-tumor tissues	Surgical resection	Recurrence versus no recurrence within 2 years post surgery	Mostly HBV	2D-DIGE, MALDI-TOF/MS		IHC, TMA
Taoka et al. (2014)	Tumor, non-tumor, and normal liver tissues	Surgical resection	Recurrence within 2 years (ER), no recurrence after 2 years (LR), adjacent non-tumor tissue, or normal liver tissue	Mixed	PROTOMAP profiling method (SDS-PAGE, LC-MS/MS)	Signal transducer and activator of transcription 1-alpha/beta (↑)	Western blot
Huang et al. (2014)	Tumor tissues	Surgical resection	Recurrence within 6 months, recurrence between 6 and 12 months, and no recurrence within 2 years post surgery	HBV	iTRAQ-2DLC-MS/MS	Alpha-methylacyl-CoA racemase (↓) Protein S100-A12 (↓)	IHC

HBV hepatitis B virus, *HCV* hepatitis C virus, *2D-DIGE* 2-dimensional-difference gel electrophoresis, *MALDI-TOF/MS* matrix-assisted laser desorption ionization time of flight, *LC-MS/MS* liquid chromatography-tandem mass spectrometry, *cICAT* cleavable isotope-coded affinity tags, *iTRAQ* isobaric tags for relative and absolute quantitation, *IHC* immunohistochemistry, *qRT-PCR* quantitative real-time-polymerase chain reaction, *SELDI-TOF-MS* surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, *TMA* tissue microarray

clinical validation. Yi et al. applied conventional 2-DE on tissue lysates of matched tumor and noncancerous tissues of hepatitis B virus (HBV)-positive patients for discovery of recurrence-related biomarkers (Yi et al. 2008). Stress-70 protein was determined to have the highest discriminatory capacity (sensitivity = 90.9%, specificity = 71.4%) in distinguishing patients with early recurrence within 1 year of surgery. Its expression was positively correlated to increasing metastatic capacity in HCC cell lines and associated with advanced tumor stage and positive venous invasion. Huang et al. used iTRAQ-based LC-MS/MS approach to interrogate the tumor proteome of HCC patients with no recurrence, early recurrence, and late recurrence (Huang et al. 2014). Alpha-methylacyl-CoA racemase (AMACR) and S100-A12 were found to be reversely regulated in patients with early and late recurrence, of which both proteins were downregulated in early recurrence and upregulated in late recurrence with respect to patients with no recurrence. Low AMACR expression was subsequently determined to be correlated with poor prognosis of HCC patients by tissue microarray analysis (Xu et al. 2014).

Some studies might propose algorithms for prediction of recurrence risk based on data from the discovery set, and the performance of these algorithms would be validated on a separate cohort. This approach was employed by Yookoo et al., who applied 2D-DIGE analysis of tumor tissue lysates to determine proteins that can separate patients with different recurrence status (Yokoo et al. 2007). A panel of 23 protein spots was identified, and this panel successfully grouped 16 out of 17 patients in validation cohort according to their recurrence status. However, the validation method involves the use of 2D-DIGE to obtain the proteome map. This requires high level of technical competence; hence, it may be challenging to translate the above results into the clinic. Similarly, Tan et al. applied 2D-DIGE to identify biomarkers that can distinguish between stage I patients with early recurrence leading to aggressive metastatic HCC and patients with no recurrence (Tan et al. 2014). Eight proteins were found to be differentially expressed, of which the expression of three proteins, namely, heat shock protein 70 kDa protein 1A/1B, argininosuccinate synthase, and UTP-glucose-1-phosphate uridylyltransferase, was validated using tissue microarray. An algorithm was generated based on the data obtained from tissue microarray for prediction of risk of aggressive metastatic recurrence. In another study, serum samples of patients who undergo microwave ablation for therapy were used for prediction of early HCC recurrence (Cao et al. 2013). The SELDI-TOF-MS platform was used, and nine different protein peaks were significantly regulated. Three protein peaks with m/z values of 7,787, 6,858, and 6,646 were used to generate a decision tree which has a sensitivity of 85.7% and specificity of 88.9%. The ease of translation combined with the use of minimally invasive biological fluid makes the use of SELDI-TOF-MS an attractive method for biomarker discovery. However, the lack of information on the proteins associated with the differentially regulated peaks makes it difficult for further functional studies to be performed. Hence, caution must be taken when using SELDI-TOF-MS as the discovery platform, as limited information can be obtained from the study.

Integration of Cancer-Associated Factors in the Study Design of Proteomic Studies

Some of the studies choose proteins for functional and clinical validation based on their biological relevance to recurrence or cancer progression. Two of the studies that identified protein biomarkers associated with early recurrence after liver transplantation took this approach. Bai et al. used cICAT LC-MS/MS to quantitate relative protein expression changes between these early recurrence and non-recurrence patients (Bai et al. 2009). Calpain small subunit 1 (Capn4) was selected for further functional validation based on its interaction with metastasis-related proteins, as well as the correlation of Capn4 expression to HCC cell lines with different metastatic potential. Knockdown of Capn4 resulted in decreased wound healing, migration, and invasion of highly metastatic MHCC97H cell line. High expression of Capn4 is associated with decreased survival rate and tumor factors associated with metastasis, such as the presence of venous invasion and the absence of tumor encapsulation. In another study, Cheng et al. used 2-DE to interrogate the tumor proteome of HCC patients with and without early recurrence (Cheng et al. 2011). N-myc downstream-regulated gene 1 protein (NDRG1) was selected for further validation as it was reported to be a downstream target of hypoxia-inducible factor 1α. Upregulation of NDRG1 in tumor tissues was validated via Western blot, and siRNA knockdown of NDRG1 resulted in decreased proliferation, migration, and invasion of HepG2 cells. High NDRG1 expression is also associated with poor prognosis and tumor factors such as increased tumor size and the presence of vascular invasion. These two studies had demonstrated the involvement of the proteins associated with HCC recurrence in metastasis-related processes and could be targets for prophylactic treatment.

In the other studies, the samples were selected based on pathological factors associated with HCC progression and prognosis. Orimo et al. used tumor tissues with different histological differentiation status and compared their proteomes to adjacent non-tumor tissues and normal liver tissues by 2D-DIGE (Orimo et al. 2008). Twenty-six differentially expressed proteins successfully segregated the patients according to the differentiation status (Orimo et al. 2008). Microtubule-associated protein RP/EB family member 1 (EB1) was selected for further validation based on its functional association with c-Myc, RhoA, and cdc42 and its high expression in poorly differentiated tumors. EB1 expression was subsequently determined to be an independent prognostic factor for recurrence and survival of patients after resection. Kanamori et al. analyzed four early HCC and non-HCC tissues from two patient cases to identify proteins involved in HCC progression by label-free LC-MS analysis (Kanamori et al. 2011). Sixty-one differentially regulated proteins were found, of which talin-1 was chosen for validation as many cytoskeletal proteins were reported to be associated with HCC progression. They showed that high talin-1 expression was associated with poor differentiation, the presence of portal vein invasion, and intrahepatic metastasis.

Lastly, the understanding of biological processes involved in HCC progression could affect the proteomic tool used in the study. Excessive proteolysis is observed

in pathological events such as cancer (van Kempen et al. 2006). Levels of aberrant proteolytic fragments may be correlated to the severity of the disease. As such, Taoka et al. attempted to use the PROTOMAP profiling approach to identify proteins with differential expression levels of normal and proteolytic fragments in patients with different recurrence status (Taoka et al. 2014). This approach involves the separation of tissue lysates by molecular weight via SDS-PAGE, cutting of gel pieces according to molecular weight, in-gel digestion, and LC-MS identification of the proteins. In addition, the protein abundance is estimated by emPAI parameters, which is subsequently used to generate heat map "peptographs." In these peptographs, the identified peptides for a given protein were plotted vertically (from N- to C-terminal), and the SDS migration of the protein and its fragments were plotted horizontally. The abundance of the peptide was indicated by the intensity of the heat map in the peptographs. Forty-six upregulated proteins and 41 downregulated proteins were identified, of which Western blot analysis clearly demonstrated the specific proteolysis of signal transducer and activator of transcription 1 (STAT1) in tumor tissue lysates of HCC patients with early recurrence.

Protein Biomarkers that Demonstrated Consistent Regulation in Multiple Publications

Typically, proteomic studies identified many proteins that were dysregulated, but only a minority were selected for validation due to limited amounts of biological samples, availability and cost of commercial antibodies for validation, and lack of novelty and knowledge of the biological function of these proteins. There are proteins that have been "validated" by being consistently found to be dysregulated in various studies and thus may be considered as potential markers. The list of proteins that were consistently regulated in two or more publications is shown in Table 2. Interestingly, several cytoskeletal proteins, heat shock proteins, and transcription regulators are upregulated in HCC recurrence. This could be linked to morphological changes, increased proliferation, and survival of tumor cells, leading to poor prognosis.

The liver is one of the main metabolic organs in the body; thus, it is not surprising that dysregulated metabolic pathways contribute to HCC progression and recurrence. Upregulation of galactokinase and downregulation of fructose-1,6-bisphosphate expression might result in the channeling of glucose and derivatives into the glycolytic pathway, which is the main source of energy production for tumor cells in the phenomenon termed the "Warburg effect" (Warburg 1956). The decrease in expression of enzymes involved in folate and amino acid metabolism may reflect a general reduction in liver function. In particular, downregulation of S-adenosylmethionine synthase, the enzyme essential for formation of S-adenosylmethionine (AdoMet) in the methylation cycle of the liver, is reported in two independent publications. Chronic decrease in AdoMet results in the development of HCC in mouse models (Martinez-Chantar et al. 2002), and proteomic studies by Liang et al. and Sun et al. demonstrated the downregulation of S-adenosylmethionine synthase in HBV-associated HCC tissues, suggesting the

Table 2 Proteins with consistent regulation patterns reported in multiple publications. This table shows the list of proteins that have similar regulation patterns reported in two or more publications. The expression levels of these proteins are not validated in the respective publications. The proteins are ordered according to their regulation patterns and key functions (↑ = upregulated in recurrence, ↓ = downregulated in recurrence)

UniProt accession number	Protein name	Key function	Regulation	References
P12004	Proliferating cell nuclear antigen	DNA replication and repair	↑	Orimo et al. (2008)
				Cheng et al. (2011)
P61978	Heterogeneous nuclear ribonucleoprotein K	Nucleic acid binding	↑	Bai et al. (2009)
				Taoka et al. (2014)
P02751	Fibronectin	Cell adhesion and cell motility	↑	Bai et al. (2009)
				Taoka et al. (2014)
P60709	Actin, cytoplasmic 1	Cytoskeletal protein	↑	Cheng et al. (2011)
				Kanamori et al. (2011)
P07437	Tubulin beta chain	Cytoskeletal protein	↑	Cheng et al. (2011)
				Kanamori et al. (2011)
P04792	Heat shock protein beta-1	Molecular chaperone	↑	Cheng et al. (2011)
				Taoka et al. (2014)
P51570	Galactokinase	Carbohydrate metabolism	↑	Tan et al. (2014)
				Taoka et al. (2014)
P09467	Fructose-1,6-bisphosphatase 1	Gluconeogenesis	↓	Orimo et al. (2008)
				Taoka et al. (2014)
O75891	Cytosolic 10-formyltetrahydrofolate dehydrogenase	Folate metabolism	↓	Bai et al. 2009
				Cheng et al. (2011)
O95954	Formimidoyltransferase-cyclodeaminase	Amino acid and folate metabolism	↓	Orimo et al. (2008)
				Cheng et al. (2011)

(*continued*)

Table 2 (continued)

UniProt accession number	Protein name	Key function	Regulation	References
Q00266	S-Adenosylmethionine synthase isoform type-1	Amino acid metabolism	↓	Orimo et al. (2008)
				Cheng et al. (2011)
P32754	4-Hydroxyphenylpyruvate dioxygenase	Amino acid metabolism	↓	Orimo et al. (2008)
				Cheng et al. (2011)
				Taoka et al. (2014)
O95154	Aflatoxin B1 aldehyde reductase member 3	Aldehyde metabolism, detoxification)	↓	Orimo et al. (2008)
				Taoka et al. (2014)
P30084	Enoyl-CoA hydratase, mitochondrial	Lipid metabolism	↓	Orimo et al. (2008)
				Tan et al. (2014)
P00441	Superoxide dismutase [Cu-Zn]	Antioxidant	↓	Yokoo et al. (2007)
				Cheng et al. (2011)
P30039	Phenazine biosynthesis-like domain-containing protein	Unknown	↓	Orimo et al. (2008)
				Cheng et al. (2011)

involvement of decreased AdoMet levels in HCC progression (Liang et al. 2005; Sun et al. 2007). Increased oxidative stress has also been correlated to an increased risk of HCC recurrence (Suzuki et al. 2013), which may be reflected by decreased levels of antioxidative enzymes such as superoxide dismutase. Overall, the list of proteins indicated the changes in expressions of these proteins that promote HCC progression are also involved in increased risk of recurrence, and thus they could be potential prognostic markers for HCC.

Future Perspectives

Despite the numerous publications that used omics-based methods in identification of predictive biomarkers for HCC recurrence, none of the suggested markers are currently used in the clinic. Furthermore, current treatment modalities are limited to early-stage cancers, with sorafenib being the only treatment available for late-stage

HCC patients. Currently, there are no medications available that show efficacy in preventing HCC recurrence. In the following sections, the improvements that can be made in future biomarker discovery and clinical translation will be discussed.

Selection of Patients for the Discovery Phase

Standardization of inclusion criteria for the samples used in the discovery phase would yield more meaningful results for clinical use. Improvements in imaging techniques have resulted in an increased numbers of HCC patients diagnosed in the early stages. The tissue samples analyzed in the discovery phase should preferably include early-stage patients with well-differentiated tumors and similar etiology to minimize confounding factors that might influence the data obtained from the proteomic studies. Subsequently, patient cohorts with mixed etiology and tumor stage could be used during the validation phase to ascertain the applicability of the protein signatures for recurrence prediction in the clinical setting.

Use of Clonal Analysis in Determination of Intrahepatic Recurrence

The cutoff time for early recurrence is not standardized and varies between 1 and 2 years in the literature. The use of a cutoff time may not be biologically relevant, as de novo synthesis of new tumors may occur within 2 years after resection. Furthermore, in patients exhibiting multifocal HCC during recurrence, it is difficult to determine the origins of the tumors. Hence, clonal analysis of both recurrent and primary tumors should be carried out to confirm the true "relapse" of HCC. These cases may then be selected and classified as the "true" intrahepatic metastasis/relapse, and the primary tumor proteome can be compared with other HCC cases with no relapse or with multicentric occurrence. This approach has recently been applied to two patients (Miao et al. 2014), which combined the use of whole genome sequencing, RNA sequencing, and single-nucleotide polymorphism analysis to determine tumor clonality and identify differentially expressed genes and pathways between tumors arising from intrahepatic recurrence and multicentric carcinogenesis. A similar approach combining clonal analysis and the use of proteomics is recommended for future studies to minimize confounding factors introduced by inclusion of patients suffering from multicentric carcinogenesis.

Identification of Molecular Pathways and Drug Targets in Proteomic Studies

Most of the current proteomic studies focused on identification of single biomarker that can best discriminate patients with or without early recurrence. However, cancer is a heterogeneous disease resulting from accumulation of genetic

aberrations and the interaction with environmental factors; hence, a single biomarker may be insufficient for prediction of recurrence. Furthermore, changes in tumor proteome may have synergistic or redundant effects that contribute to tumor progression and survival. Redundancies in molecular pathways contribute to the failure of molecular-based therapies for cancer treatment. Hence, it may be beneficial to look at molecular and signaling pathways that are dysregulated in tumors of recurrent cancers. A combination of transcriptomic and proteomic data may provide information with regard to the overexpression of specific transcription factors, which can be linked to the change in expression levels of downstream protein targets. This might allow us to identify suitable protein targets within the pathway such as receptor molecules and kinases that are more amenable for targeting by small molecules.

Identification and Validation of Potential Biomarkers in Serum/Plasma

Current efforts have been placed in identification of protein biomarkers using tissue samples. However, it is not feasible to perform repeated sampling of liver tissues for surveillance. Biofluids such as serum and plasma are good sources for proteomic analysis as they can be obtained from patients with minimal invasiveness. Most of the clinically approved biomarkers for cancer detection and monitoring are serum based (Fuzery et al. 2013). Hence, despite the challenges faced by serum and plasma proteomics (Anderson and Anderson 2002), it may be worthwhile to use these biofluids as samples for biomarker discovery. Alternatively, if the proteomic studies on tissues identified known secreted proteins, their expression levels in serum/plasma could differ. This could result in the development of a serum-based biomarker which could be translated into clinical practices.

Push for Better Commercial Antibodies for Validation of Candidate Biomarkers

Validation of proteomic data usually involves the use of antibody-based detection methods, such as immunohistochemistry and Western blotting, in determination of expression levels of protein of interest in a separate sample cohort. The availability and quality of antibodies are of upmost importance for obtaining reliable information. The Human Protein Atlas project was initiated to fulfill this unmet need by generating high-quality antibodies as well as validating existing commercial antibodies (Berglund et al. 2008; Ponten et al. 2008; Uhlen et al. 2015). The Human Protein Atlas portal is publically available (www.proteinatlas.org) and showcases spatial expression of proteins in different human tissues and cancer types, as well as cancer cell lines. This is a big step to improve the quality and number of antibodies available for validation purposes.

Alternative Validation Methods

Improvements in mass spectrometry have led to the possibility of reproducible quantitation of proteins across different types of biological samples and a wide dynamic range of protein detection. This can be achieved by targeted methods such as selected reaction monitoring (SRM), in which precursor/fragment ion pairs that uniquely represent a peptide are first selected and protein quantitation is obtained by comparison of signals obtained from the transition signals to a suitable reference. The analysis method could also be applied to data from SWATH-MS. Similar techniques are currently in use for routine monitoring of small molecules in clinical labs, which would aid the transition from the bench to the clinic.

Other Potential Applications of Proteomics in Cancer

In the review, we have highlighted the use of proteomics in identification of predictive markers for early HCC recurrence. Despite the progress in separation and mass spectrometry technologies, the number of clinically approved biomarkers arising from proteomic studies is dismal. OVA1 remains as the one and only clinically approved panel of five protein biomarkers for detection of ovarian cancer. These five proteins include apolipoprotein A1, prealbumin, transferrin, beta-2 microglobulin, and CA 125II, of which the first four proteins were identified by SELDI-TOF-MS (Zhang et al. 2004). We believe that the combination of a good study design, coupled with the rapid improvements in mass spectrometry technology, would result in a panel of biomarkers with high sensitivity and specificity for prognosis of HCC.

Proteomics can also be applied in understanding the mechanisms of existing cancer drugs. This would unravel the mechanisms of drug's action and drug resistance as well as identify predictive biomarkers for drug response. Such studies had been performed on genistein (Narasimhan et al. 2015), paclitaxel (Xu et al. 2015), and sorafenib (Yeh et al. 2015). This forms the basis of personalized medicine, where medications would be matched to individual cancer patients based on the characteristics of the tumor proteome and the mechanism of drugs' action.

Summary Points

- Recurrence is the main cause of death for patients who received curative treatment for hepatocellular carcinoma.
- Recurrence can be classified into two main forms, namely, intrahepatic metastasis and multicentric carcinogenesis.
- Clonal analysis indicated that the recurrent tumors from intrahepatic metastasis originate from the primary tumor, and these tumors are usually more aggressive and lead to poor prognosis.

- Most of the proteomic studies interrogate the proteome of the primary tumor to unravel biomarkers for prediction of early recurrence.
- Proteomic techniques used in the studies include gel-based, liquid chromatography-based, and chip-based platforms.
- Candidate biomarkers identified in these studies have diverse molecular functions. They include heat shock proteins, cytoskeletal proteins, and metabolic enzymes.
- Functional validations in cell lines implicate the overexpression of these proteins in promoting metastasis and cell proliferation.
- Improvements can be made in the study design for discovery of predictive biomarkers. This includes the careful selection of patients based on tumor staging and inclusion of clonal analysis of the primary and recurrent tumor/s.

References

Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics. 2002;1:845–67.

Arentz G, Weiland F, Oehler MK, Hoffmann P. State of the art of 2D DIGE. Proteomics Clin Appl. 2015;9:277–88.

Bai DS, Dai Z, Zhou J, Liu YK, Qiu SJ, Tan CJ, Shi YH, Huang C, Wang Z, He YF, Fan J. Capn4 overexpression underlies tumor invasion and metastasis after liver transplantation for hepatocellular carcinoma. Hepatology. 2009;49:460–70.

Berglund L, Bjorling E, Oksvold P, Fagerberg L, Asplund A, Szigyarto CA, Persson A, Ottosson J, Wernerus H, Nilsson P, Lundberg E, Sivertsson A, Navani S, Wester K, Kampf C, Hober S, Ponten F, Uhlen M. A genecentric human protein atlas for expression profiles based on antibodies. Mol Cell Proteomics. 2008;7:2019–27.

Cao XL, Li H, Yu XL, Liang P, Dong BW, Fan J, Li M, Liu FY. Predicting early intrahepatic recurrence of hepatocellular carcinoma after microwave ablation using SELDI-TOF proteomic signature. PLoS One. 2013;8:e82448.

Cheng J, Xie HY, Xu X, Wu J, Wei X, Su R, Zhang W, Lv Z, Zheng S, Zhou L. NDRG1 as a biomarker for metastasis, recurrence and of poor prognosis in hepatocellular carcinoma. Cancer Lett. 2011;310:35–45.

Cheng Z, Yang P, Qu S, Zhou J, Yang J, Yang X, Xia Y, Li J, Wang K, Yan Z, Wu D, Zhang B, Huser N, Shen F. Risk factors and management for early and late intrahepatic recurrence of solitary hepatocellular carcinoma after curative resection. HPB (Oxford). 2015;17:422–7.

Choe L, D'Ascenzo M, Relkin NR, Pappin D, Ross P, Williamson B, Guertin S, Pribil P, Lee KH. 8-plex quantitation of changes in cerebrospinal fluid protein expression in subjects undergoing intravenous immunoglobulin treatment for Alzheimer's disease. Proteomics. 2007;7:3651–60.

Cucchetti A, Piscaglia F, Caturelli E, Benvegnu L, Vivarelli M, Ercolani G, Cescon M, Ravaioli M, Grazi GL, Bolondi L, Pinna AD. Comparison of recurrence of hepatocellular carcinoma after resection in patients with cirrhosis to its occurrence in a surveilled cirrhotic population. Ann Surg Oncol. 2009;16:413–22.

Du ZG, Wei YG, Chen KF, Li B. Risk factors associated with early and late recurrence after curative resection of hepatocellular carcinoma: a single institution's experience with 398 consecutive patients. Hepatobiliary Pancreat Dis Int. 2014;13:153–61.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136:E359–86.

Franssen B, Jibara G, Tabrizian P, Schwartz ME, Roayaie S. Actual 10-year survival following hepatectomy for hepatocellular carcinoma. HPB (Oxford). 2014;16:830–5.

Fuzery AK, Levin J, Chan MM, Chan DW. Translation of proteomic biomarkers into FDA approved cancer diagnostics: issues and challenges. Clin Proteom. 2013;10:13.

Gillet LC, Navarro P, Tate S, Rost H, Selevsek N, Reiter L, Bonner R, Aebersold R. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. Mol Cell Proteomics. 2012;11(O111):016717.

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol. 1999;17:994–9.

Huang X, Zeng Y, Xing X, Zeng J, Gao Y, Cai Z, Xu B, Liu X, Huang A, Liu J. Quantitative proteomics analysis of early recurrence/metastasis of huge hepatocellular carcinoma following radical resection. Proteome Sci. 2014;12:22.

Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics. 2005;4:1265–72.

Issaq HJ, Veenstra TD, Conrads TP, Felschow D. The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. Biochem Biophys Res Commun. 2002;292:587–92.

Kanamori H, Kawakami T, Effendi K, Yamazaki K, Mori T, Ebinuma H, Masugi Y, Du W, Nagasaka K, Ogiwara A, Kyono Y, Tanabe M, Saito H, Hibi T, Sakamoto M. Identification by differential tissue proteome analysis of talin-1 as a novel molecular marker of progression of hepatocellular carcinoma. Oncology. 2011;80:406–15.

Lau WY, Lai EC. Hepatocellular carcinoma: current management and recent advances. Hepatobiliary Pancreat Dis Int. 2008;7:237–57.

Liang CR, Leow CK, Neo JC, Tan GS, Lo SL, Lim JW, Seow TK, Lai PB, Chung MC. Proteome analysis of human hepatocellular carcinoma tissues by two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics. 2005;5:2258–71.

Mancuso A. Management of hepatocellular carcinoma: enlightening the gray zones. World J Hepatol. 2013;5:302–10.

Martinez-Chantar ML, Corrales FJ, Martinez-Cruz LA, Garcia-Trevijano ER, Huang ZZ, Chen L, Kanel G, Avila MA, Mato JM, Lu SC. Spontaneous oxidative stress and liver tumors in mice lacking methionine adenosyltransferase 1A. FASEB J. 2002;16:1292–4.

Miao R, Luo H, Zhou H, Li G, Bu D, Yang X, Zhao X, Zhang H, Liu S, Zhong Y, Zou Z, Zhao Y, Yu K, He L, Sang X, Zhong S, Huang J, Wu Y, Miksad RA, Robson SC, Jiang C, Zhao H. Identification of prognostic biomarkers in hepatitis B virus-related hepatocellular carcinoma and stratification by integrative multi-omics analysis. J Hepatol. 2014;61:840–9.

Morimoto O, Nagano H, Sakon M, Fujiwara Y, Yamada T, Nakagawa H, Miyamoto A, Kondo M, Arai I, Yamamoto T, Ota H, Dono K, Umeshita K, Nakamori S, Sasaki Y, Ishikawa O, Imaoka S, Monden M. Diagnosis of intrahepatic metastasis and multicentric carcinogenesis by microsatellite loss of heterozygosity in patients with multiple and recurrent hepatocellular carcinomas. J Hepatol. 2003;39:215–21.

Narasimhan K, Lee YM, Lim TK, Port SA, Han JH, Chen CS, Lin Q. Genistein exerts anti-leukemic effects on genetically different acute myeloid leukemia cell lines by inhibiting protein synthesis and cell proliferation while inducing apoptosis – molecular insights from an iTRAQ quantitative proteomics study. Oncoscience. 2015;2:111–24.

Orimo T, Ojima H, Hiraoka N, Saito S, Kosuge T, Kakisaka T, Yokoo H, Nakanishi K, Kamiyama T, Todo S, Hirohashi S, Kondo T. Proteomic profiling reveals the prognostic value of adenomatous polyposis coli-end-binding protein 1 in hepatocellular carcinoma. Hepatology. 2008;48:1851–63.

Ponten F, Jirstrom K, Uhlen M. The human protein atlas – a tool for pathology. J Pathol. 2008;216:387–93.

Poon RT, Fan ST, Wong J. Risk factors, prevention, and management of postoperative recurrence after resection of hepatocellular carcinoma. Ann Surg. 2000;232:10–24.

Portolani N, Coniglio A, Ghidoni S, Giovanelli M, Benetti A, Tiberio GA, Giulini SM. Early and late recurrence after liver resection for hepatocellular carcinoma: prognostic and therapeutic implications. Ann Surg. 2006;243:229–35.

Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics. 2004;3:1154–69.

Sherman M. Recurrence of hepatocellular carcinoma. N Engl J Med. 2008;359:2045–7.

Sun W, Xing B, Sun Y, Du X, Lu M, Hao C, Lu Z, Mi W, Wu S, Wei H, Gao X, Zhu Y, Jiang Y, Qian X, He F. Proteome analysis of hepatocellular carcinoma by two-dimensional difference gel electrophoresis: novel protein markers in hepatocellular carcinoma tissues. Mol Cell Proteomics. 2007;6:1798–808.

Suzuki Y, Imai K, Takai K, Hanai T, Hayashi H, Naiki T, Nishigaki Y, Tomita E, Shimizu M, Moriwaki H. Hepatocellular carcinoma patients with increased oxidative stress levels are prone to recurrence after curative treatment: a prospective case series study using the d-ROM test. J Cancer Res Clin Oncol. 2013;139:845–52.

Tan GS, Lim KH, Tan HT, Khoo ML, Tan SH, Toh HC, Ching Ming Chung M. Novel proteomic biomarker panel for prediction of aggressive metastatic hepatocellular carcinoma relapse in surgically resectable patients. J Proteome Res. 2014;13:4833–46.

Taoka M, Morofuji N, Yamauchi Y, Ojima H, Kubota D, Terukina G, Nobe Y, Nakayama H, Takahashi N, Kosuge T, Isobe T, Kondo T. Global PROTOMAP profiling to search for biomarkers of early-recurrent hepatocellular carcinoma. J Proteome Res. 2014;13:4847–58.

Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson A, Kampf C, Sjostedt E, Asplund A, Olsson I, Edlund K, Lundberg E, Navani S, Szigyarto CA, Odeberg J, Djureinovic D, Takanen JO, Hober S, Alm T, Edqvist PH, Berling H, Tegel H, Mulder J, Rockberg J, Nilsson P, Schwenk JM, Hamsten M, Von Feilitzen K, Forsberg M, Persson L, Johansson F, Zwahlen M, Von Heijne G, Nielsen J, Ponten F. Proteomics. Tissue-based map of the human proteome. Science. 2015;347:1260419.

Unlu M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis. 1997;18:2071–7.

Van Kempen LC, De Visser KE, Coussens LM. Inflammation, proteases and cancer. Eur J Cancer. 2006;42:728–34.

Voss T, Haberl P. Observations on the reproducibility and matching efficiency of two-dimensional electrophoresis gels: consequences for comprehensive data analysis. Electrophoresis. 2000;21:3345–50.

Wang B, Xia CY, Lau WY, Lu XY, Dong H, Yu WL, Jin GZ, Cong WM, Wu MC. Determination of clonal origin of recurrent hepatocellular carcinoma for personalized therapy and outcomes evaluation: a new strategy for hepatic surgery. J Am Coll Surg. 2013;217:1054–62.

Warburg O. On the origin of cancer cells. Science. 1956;123:309–14.

Washburn MP, Wolters D, Yates 3rd JR. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol. 2001;19:242–7.

Wu JC, Huang YH, Chau GY, Su CW, Lai CR, Lee PC, Huo TI, Sheen IJ, Lee SD, Lui WY. Risk factors for early and late recurrence in hepatitis B-related hepatocellular carcinoma. J Hepatol. 2009;51:890–7.

Xu B, Cai Z, Zeng Y, Chen L, Du X, Huang A, Liu X, Liu J. alpha-Methylacyl-CoA racemase (AMACR) serves as a prognostic biomarker for the early recurrence/metastasis of HCC. J Clin Pathol. 2014;67:974–9.

Xu H, Dephoure N, Sun H, Zhang H, Fan F, Liu J, Ning X, Dai S, Liu B, Gao M, Fu S, Gygi SP, Zhou C. Proteomic profiling of paclitaxel treated cells identifies a novel mechanism of drug resistance mediated by PDCD4. J Proteome Res. 2015;14:2480–91.

Yeh CC, Hsu CH, Shao YY, Ho WC, Tsai MH, Feng WC, Chow LP. Integrated SILAC and iTRAQ quantitative proteomic analysis identifies galectin-1 as a potential biomarker for predicting sorafenib resistance in liver cancer. Mol Cell Proteomics. 2015;14:1527–45.

Yi X, Luk JM, Lee NP, Peng J, Leng X, Guan XY, Lau GK, Beretta L, Fan ST. Association of mortalin (HSPA9) with liver cancer metastasis and prediction for early tumor recurrence. Mol Cell Proteomics. 2008;7:315-–25.

Yokoo H, Kondo T, Okano T, Nakanishi K, Sakamoto M, Kosuge T, Todo S, Hirohashi S. Protein expression associated with early intrahepatic recurrence of hepatocellular carcinoma after curative surgery. Cancer Sci. 2007;98:665–73.

Zhang Z, Bast Jr RC, Yu Y, Li J, Sokoll LJ, Rai AJ, Rosenzweig JM, Cameron B, Wang YY, Meng XY, Berchuck A, Van Haaften-Day C, Hacker NF, De Bruijn HW, Van Der Zee AG, Jacobs IJ, Fung ET, Chan DW. Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. Cancer Res. 2004;64:5882–90.

Zhang X, Liu S, Shen C, Wu Y, Zhang, L, Chen X, Lu F. DNA methylation consistency implicates the primary tumor cell origin of recurrent hepatocellular carcinoma. Epigenomics. 2015;7:589–92.

Biomarkers to Monitor Graft Function Following Liver Transplantation

9

Cornelia J. Verhoeven, Luc J. W. van der Laan, Jeroen de Jonge, and Herold J. Metselaar

Contents

C.J. Verhoeven • L.J.W. van der Laan
Department of Surgery, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands
e-mail: c.j.verhoeven@erasmusmc.nl; l.vanderlaan@erasmusmc.nl

J. de Jonge
Department of Surgery, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands

Department of Surgery, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands
e-mail: j.dejonge.1@erasmusmc.nl

H.J. Metselaar (✉)
Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands
e-mail: h.j.metselaar@erasmusmc.nl

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_20

Abstract

Liver transplantation (LT) has become the only curative treatment for end-stage liver disease. Patient survival has improved drastically over the years, but poor initial graft quality and complications following transplantation still limit patient and graft survival. Monitoring and evaluation of graft quality during follow-up is achieved by routine biomarker measurements in recipients' blood, starting directly following surgery and in the months and years thereafter. This allows clinicians to early detect complications following LT, like early allograft dysfunction and biliary complications. They are also used as a tool for deciding on further diagnostics or interventions. Classic biomarkers are able to assess liver injury (aspartate and alanine aminotransferase, lactate dehydrogenase), biliary injury and obstruction (gamma-glutamyl transferase, alkaline phosphatase), and liver function (albumin, bilirubin, prothrombin time). Novel genetic markers such as microRNAs also show potential as more accurate or specific biomarker for various types of injury and functions. Some of these serum biomarkers were shown to be promising in predicting disease or severity of injury when measured in bile, though widespread implementation in clinical practice is not implemented yet. Therefore, liver biopsy remains the gold standard for diagnosing acute cellular rejection, even with less invasive serum biomarkers that are currently available. Future applications of biomarkers should enable early assessment of marginal graft function when applied to preservation solution in both simple cold storage and during ex situ machine perfusion. In the future, these developments could help to increase the donor pool for LT by optimizing and allocating grafts based on favorable biomarker profiles from donors with unfavorable clinical characteristics.

Keywords

Serum markers • Transaminases • Complications • Graft dysfunction • Biliary strictures • Cholestasis • Recurrence of disease • microRNAs • Machine perfusion • Risk factors

List of Abbreviations

ACR	Acute cellular rejection
AFP	Alpha fetoprotein
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AS	Anastomotic biliary stricture
AST	Aspartate aminotransferase
CA 19-9	Cancer antigen 19-9
CCA	Cholangiocarcinoma
CDmiR	Cholangiocyte-derived miRNA
DCD	Donation after circulatory death
EAD	Early allograft dysfunction
ERCP	Endoscopic retrograde cholangiopancreatography
GGT	Gamma-glutamyl transferase
HCC	Hepatocellular carcinoma
HDmiR	Hepatocyte-derived miRNA
LT	Liver transplantation
MiRNA	microRNA
MP	Machine perfusion.
MRCP	Magnetic resonance cholangiopancreatography
mRNA	Messenger RNA
NAS	Non-anastomotic biliary stricture
PNF	Primary nonfunction
PSC	Primary sclerosing cholangitis
SNP	Single nucleotide polymorphism

Key Facts of microRNAs

- MicroRNAs (also called miRNAs or miRs) are 20–23 nucleotide-long, hairpin-shaped RNA. Up to 30% of the human genes is regulated by miRNAs via inhibition of mRNA translation.
- A single miRNA is responsible for the regulation of multiple genes.
- The first reports on the presence of miRNAs in *Caenorhabditis elegans* date from 2001, and since then, over a 1,000 different miRNAs have been discovered in mammals.
- Various cell types express distinct sets of miRNAs that are related to metabolism, oncology, endocrinology, the vascular system, and infection.
- Tissue-abundant miRNAs are released from cells into the circulation and other body fluids under different (patho)physiological conditions via active and passive mechanisms.

– In contrast to mRNA, extracellular miRNA is protected from degradation in fluids, making them attractive for noninvasive biomarker research.

Definitions of Words and Terms

Anastomotic stricture (AS)	Isolated benign tapering of the biliary anastomosis following LT.
Cholangiocarcinoma (CCA)	Malignancy of the hepatic bile ducts and cholangiocytes.
Cholestasis	Accumulation of bile due to obstruction flow to the duodenum or altered bile composition.
Donation after brain death (DBD)	Procurement of donor organs after disappearance of brain stem functions (brain death), while the circulation is still intact. Organs are usually of better quality compared to DCD.
Donation after circulatory death (DCD)	Procurement of donor organs after circulatory arrest of the donor. Associated with warm-ischemic injury of organs.
Early allograft dysfunction (EAD)	Poor graft function in the first week post-LT, based on AST or ALT >2,000 IU/L, or total bilirubin serum levels >10 μg/L on day 7 post-LT, or INR >1.6 on day 7 post-LT.
Hepatocellular carcinoma (HCC)	Malignancy of liver parenchyma and hepatocytes.
MicroRNAs	Small, noncoding RNAs involved in posttranscriptional gene regulation. Potential novel biomarkers.
Non-anastomotic strictures (NAS)	Benign tapering of the intrahepatic and (perihilar)-extrahepatic bile ducts following LT.
Preservation	Storage of organs at cold temperature and suitable fluids to prevent deterioration of the grafts, for optimal quality and functioning following transplantation.
Primary sclerosing cholangitis (PSC)	Autoimmune disease in which there is a progressive fibrosis of the intra- and extrahepatic bile ducts.

Introduction

The liver is the largest visceral and most multifunctional organ of the human body. It produces and drains bile, which is responsible for digestion. Furthermore, the liver metabolizes glucose, proteins like albumin and coagulation factors, amino acids, and lipids. Detoxification is achieved by the breakdown of hormones like insulin and drugs. Cells in the livers' reticuloendothelial system are responsible for immunological effects and protection against certain antigens (Burroughs and Westaby

2005). This enumeration describes only part of all liver functions but also illustrates the livers' diverse and essential role for the body. Under stable conditions, the liver has 60–70% overcapacity. This allows for resection in healthy individuals of up to 70% of liver volume (Kishi et al. 2009). After such surgery, the liver will regenerate to its normal volume within weeks. However, an absent liver function due to acute liver failure or chronic end-stage liver disease is not compatible with life and can only be cured by liver transplantation (LT).

It took 4 years for Thomas Starzl to perform the first successful LT in human in 1967, after several unsuccessful attempts since 1963, with most patients dying on the operation table (Starzl et al. 1963, 1968). Still, the first LT series in human reported a 1-year survival rate of only 25%, illustrating the complex surgical technique and severe complications that could occur early following LT in those days. One of the major complications limiting patient and graft survival was acute rejection of the transplanted organ against the recipient. A decade later, survival rates of LT recipients improved drastically after Sir Roy Calne introduced cyclosporine, an immunosuppressant drug, into the clinic (Calne et al. 1979).

Nearly 50 years later, LT is regarded standard treatment for end-stage liver disease and performed worldwide in various populations suffering from different pathologies. Because of optimized surgical techniques and immunosuppressant regimens, graft survival can now reach beyond 20 years with excellent graft function in some recipients (Jain et al. 2000). This has also led to an expansion of the designated indications for LT; on-going trials investigate the benefit of LT in selected patients with cholangiocarcinoma (Darwish Murad et al. 2012a), hepatocellular carcinoma (Mazzaferro et al. 1996), and colorectal liver metastases (Dueland et al. 2015). However, while the list of patients awaiting LT is getting longer, the number of transplantable organs remains scarce. Moreover, the quality of transplantable organs is deteriorating due to increasing donor age, liver steatosis, viral hepatitis of the donor, and prolonged ischemia times following donation after circulatory death (DCD) (Durand et al. 2008). All these factors can cause a wide range of complications threatening graft and patient survival following LT. Early complications mainly consist of infections, graft primary nonfunction (PNF), early allograft dysfunction (EAD), biliary complications (i.e., leakage and anastomotic and non-anastomotic biliary strictures), and acute rejection. Besides biliary complications, other complications at the intermediate and long-term usually consist of recurrence of liver disease that initially required LT (like hepatitis C viral infection and primary sclerosing cholangitis), the development of malignancies, chronic rejection, and liver fibrosis (Verhoeven et al. 2014).

In order to discover these complications in LT recipients timely, monitoring of graft function with suitable biomarkers is required. Routine monitoring of minimally or noninvasive biomarkers enables early recognition of complications to which physicians can adapt their medical policy. Two examples are to obtain histology in the case of suspicion of allograft rejection or to perform imaging/endoscopic treatment in the case of suspicion of biliary complications. Therefore, LT recipients are subjected to protocol (blood) measurements depending on their clinical status during follow-up, varying from daily monitoring at the intensive care unit directly after

surgery to yearly routine measurements at the outpatient clinic. Different patients and underlying diseases require personalized or precision monitoring with established biomarkers in liver disease.

The following paragraphs provide an outline on the definition of biomarkers in the field of LT and the different types of biomarkers that are used in clinical practice for short- and long-term monitoring of graft function. Finally, potentially interesting novel biomarkers are discussed, and recommendations are given regarding future applications of biomarkers in the context of LT.

Definition of Biomarkers in Liver Transplantation

The term "biomarker," an amalgamation of the words "biological marker," was defined in 1998 by a working group of the National Institutes of Health, describing it as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group 2001). Since that time, however, multiple other definitions have been introduced that further expanded the interpretation of the term biomarker. This was, for instance, done by a collaboration of the World Health Organization, the United Nations, and the International Labor Organization, who defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease"(WHO 2001). Based on the descriptions above, one can conclude that biomarkers can be used to measure the effect of treatment as well as predict or be related to a clinical endpoint. Biomarkers are also increasingly being used as a primary or secondary outcome measure in experimental or clinical studies and therefore sometimes applied as a surrogate endpoint (Strimbu and Tavel 2010). Especially in LT, definitions like EAD or PNF are mainly defined by persistently elevated transaminase levels in serum, often combined with perturbed coagulation function of the liver.

Furthermore, the previously described definitions on biomarkers allow to distinguish "dynamic" markers from "static" markers. In the context of LT, dynamic markers are usually molecular markers and liver enzymes that can be measured in serum and which levels fluctuate depending on the functional status or degree of injury of the liver graft. As an example, immediately after LT, ischemia-reperfusion injury of the graft causes elevation of serum aspartate and alanine aminotransferase levels (AST, ALT) above 200 IU/L, while a more gradual rise in gamma-glutamyl transferase (GGT) or alkaline phosphatase (ALP) starts approximately 24–48 h after LT (Fig. 2). When a patient has been transplanted because of viral hepatitis as underlying pathology, routine measurements of viral load during follow-up are part of regular clinical practice. This is because of the reasonable chance of recurrence of disease in the new liver graft (Al-Hamoudi et al. 2015). Depending on the type of complication, treating the cause will ultimately result in normalization of

serum levels of dynamic markers. Therefore, dynamic markers are variable markers that can be suitable for determining whether treatment or interventions are successful.

Static markers on the other hand are less subjected to change by the (patho) physiological status of the liver graft. One could think of genetic polymorphisms like single nucleotide polymorphisms (SNPs) of either donors or recipients that are related with certain outcomes following LT. Genetic markers or SNPs are more often fixed factors that do not fluctuate or change by graft injury. However, certain polymorphisms do make LT recipients more susceptible for certain complications; several SNPs involved in the innate immunity system have been correlated to a higher incidence of severe infections post-LT (de Rooij et al. 2010). Also in recipients that were transplanted for cholestatic diseases like primary sclerosing cholangitis (PSC), certain SNPs were identified that cause earlier recurrence of severe biliary injury after LT (op den Dries et al. 2011). Because of the predicting capacity for outcome rather than their monitoring capacities, in literature, SNPs are more often referred to as "risk factors" instead of biomarkers.

A separate category of markers are histological markers or markers measured in liver biopsies. Up to a decade ago, many transplant centers monitored graft injury and rejection by evaluating histological changes in by-protocol liver biopsies during follow-up. Most dynamic markers for liver injury in serum are related to histological changes of the liver parenchyma and bile ducts (Giannini et al. 2005). However, it usually takes more time to detect histological and morphological changes in liver tissue and puncture of the liver is not harmless. Therefore, taking liver biopsies is nowadays mainly indicated to confirm suspected graft rejection and recurrence of disease or malignancy based on changes in serum biomarkers and imaging.

The next chapters will focus mainly on dynamic markers in blood and serum and the most important histological markers associate with liver injury and function following LT.

Different Biomarkers for Different Cell Types

In liver disease, biomarkers are divided in predominantly hepatocellular or cholestatic markers. Liver enzymes as AST and ALT are indicative of hepatocellular injury, while GGT and ALP reflect biliary injury or obstruction. Besides these two categories, markers of liver function are also of importance for the evaluation of graft quality, especially in the first days following LT. Very often, the liver enzymes AST and ALT are used to indirectly asses liver function. Strictly spoken they do not represent liver function but are more indicative of liver cell death. Thus, for this purpose it is more useful to analyze products that are normally metabolized or synthesized by the liver, like proteins such as albumin and certain coagulation markers. Table 1 provides an overview of classic biomarkers per cell type, injury or function, which are discussed more extensively in the following paragraphs.

Table 1 Conventional biomarkers used in liver transplantation for graft monitoring

Category	Biomarkers
Hepatocellular injury	AST, ALT, LDH
Cholangiocyte injury and cholestasis	GGT, ALP, bilirubin
Liver function	Albumin, bilirubin, PT, INR
Recurrence or new onset HCC	AFP
Recurrence or new onset CCA	CA 19-9

Biomarkers for Hepatocellular Injury

Aspartate Aminotransferase (AST)

AST is an enzyme involved in the production of proteins and catabolization of amino acids, allowing them to cross membranes and enter the citric acid cycle. In humans, AST is present in a descending concentration in the following tissues: the heart, liver, skeletal muscle, kidney, pancreas, spleen, lungs, brain, and erythrocytes. Current clinically applied techniques however do not trace tissue origin from which AST was released. Therefore, it is often necessary to involve other markers as well for the interpretation of serum AST in the clinical setting. AST can be measured in serum and plasma obtained through venipuncture, remaining stable for at least 24 h at room temperature. Halftime is approximately 12 h. Two iso-enzymes of AST can be distinguished that occur in separate cellular compartments, namely, in the cytoplasm (c-AST) and in the mitochondria (m-AST). Following mild tissue injury, particularly c-AST can be elevated in serum, while severe injury will also lead to a release of m-AST (Kirsch et al. 1984). In adult healthy individuals, the range of AST varies between 31 and 35 U/l but usually depends on sex and age (Hooijkaas et al. 2013a).

Following LT, peak AST in serum is usually reached within the first 24–48 h after surgery, sometimes being a 100-fold increased or higher. In particular when a liver graft is of poor quality, for instance, due to increased warm ischemia time, high donor age, or liver steatosis, peak AST can reach extreme values during the first week post-LT (>1,000 U/l). Although transaminase levels usually decrease quickly following LT, one should be careful with interpreting this as graft recovery. Massive hepatocellular necrosis can result in hepatic failure, which should be evaluated based on the capacity of the graft's coagulation function and bile production. Therefore, both markers for hepatocellular injury (AST, ALT) as well as cholestatic markers (ALP, GGT) and functional markers (PT, INR, albumin, bilirubin) should always be evaluated together directly following LT.

Alanine Aminotransferase (ALT)

Alanine aminotransferase (ALT) catalyzes the transfer of the amino group L-alanine to α-ketoglutarate, resulting in the production of pyruvate and L-glutamate. High

concentrations occur in the hepatocyte cytoplasm, whereas only low concentrations are found in heart and kidney tissue (Wroblewski 1958). Therefore, ALT is considered to be more liver specific compared to AST. However, because of their differences in intralobular distribution, elevation of AST levels is usually faster than ALT. Nevertheless, serum or plasma ALT has proven to be of value in the diagnostic process of various liver diseases. For instance, in acute viral hepatitis, serum ALT can quickly rise up to 20-fold its normal range, while levels of AST remain lower or show only mild increase. At the same time, the ALT/AST ratio, which is <1 in healthy individuals, becomes >1 (De Ritis et al. 2006). Chronic (viral) hepatitis results in milder elevations of AST and ALT. When levels of AST become higher than ALT, one should be aware of cellular necrosis.

Despite being markers of hepatocellular injury, biliary obstruction can also result in liver injury and therefore increased levels of AST and ALT. Furthermore, peak serum ALT levels in the first week following LT have been associated with the development of severe biliary complications (den Dulk et al. 2015). A possible explanation for this finding could lay within the distribution of ALT in the liver acinus; the bile ducts and hepatic artery are located periportally (zone 1). Ischemic injury in this zone will cause release of ALT into the serum. Zone 3 on the other hand is located pericentrally, is less oxygenated, and contains higher concentrations of AST (Giannini et al. 2005). It remains unclear whether serum levels of AST are also related to the development of biliary complications.

Just like AST, the reference value of ALT depends on sex and age but normally does not rise above 50 U/l. Be aware that halftime of ALT in plasma or serum is however longer, approximately 50 h.

Lactate Dehydrogenase (LDH)

This enzyme catalyzes the conversion of lactate into pyruvate and vice versa. Pyruvate, the product of glycolysis, is converted to lactate under anaerobic conditions. The inverse reaction takes place in the liver and results in gluconeogenesis. LDH is present in the cell cytoplasm of practically all organs in the human body, making it widely applicable but thereby also less attractive for diagnostic purposes. Also distinguishing between the five different isotypes of LDH, which differ in characteristics as halftime, does not seem to give additional diagnostic benefit. Furthermore, hemolysis can give an overestimation of LDH activity in serum. The normal range of LDH in healthy adults is <225 U/l (Hooijkaas et al. 2013b).

Despite these apparent shortcomings, LDH is still applied as a clinical biomarker in the follow-up of liver transplant recipients. Strong elevations of LDH in serum or plasma directly after liver transplantation are usually indicative for the severity of ischemia-reperfusion injury of the graft. When strong elevations of LDH prolong and are accompanied with high levels of other transaminases, one should be aware of serious complications, like hepatic artery thrombosis (Cassidy and Reynolds 1994). But experimental studies also suggest the measurement of LDH in bile to assess the

amount of biliary or cholangiocyte injury (Op den Dries et al. 2014). However, this novel application of LDH is currently not used in standard clinical practice.

Biomarkers for Biliary Obstruction or Cholestasis

Gamma-Glutamyl Transferase (GGT)

The enzyme GGT is a carboxypeptidase located in cellular membranes. It transfers gamma-glutamyl glutathione to acceptor amino acids, peptides, or water. Furthermore, it transfers amino acids across the cellular membrane. The hepatopancreatobiliary system is the largest contributor of GGT levels in serum, but high concentrations of GGT are also present in kidney tubular epithelium and prostate tissue. Lower tissues are found in the spleen, brain, and heart. The liver excretes GGT via the bile. Therefore, biliary obstructions can cause strong elevations of GGT in serum (Goldberg 1980). Together with alkaline phosphatase (ALP), GGT is useful to screen whether recipients have developed significant biliary complications following LT, in particular anastomotic and non-anastomotic strictures (AS and NAS, respectively). In contrast to ALP, GGT is not elevated in bone disease (Lum and Gambino 1972). Increased serum levels of cholestatic markers are an indication to perform further imaging to determine the cause of obstruction, generally via endoscopic retrograde cholangiopancreatography (ERCP) or via magnetic resonance cholangiopancreatography (MRCP).

In healthy adults, GGT serum levels are below 35–40 U/l. Directly following LT, levels of GGT are often not elevated but start to rise within the first postoperative days. If a recipient develops AS, levels of GGT and ALP are expected to be high, up to 400–500 U/L. Stenting of the biliary anastomosis will give a rapid normalization of serum levels, as illustrated in the right panel of Fig. 2. When a liver graft is affected by NAS, levels of GGT and ALP can strongly fluctuate, but will increase over time, since these strictures are more stubborn to treat by stents or percutaneous drains. When a mild rise in cholestatic markers is accompanied by a rise in hepatocellular markers, one should also think of (recurrence of chronic) hepatitis (Huang et al. 2014).

A paradoxical finding confirmed by multiple researchers is that higher levels of GGT early following LT are associated with improved 90-day survival in recipients, while recipients who died before the 90th postoperative day had lower GGT serum levels (Eisenbach et al. 2009). After the first 90 days, however, high levels of GGT are associated with impaired 5-year survival. It has been suggested that high levels of GGT early following surgery are the result of a proper systemic response to reactive oxygen species that are released after graft reperfusion. A different hypothesis states that the increase of GGT is correlated to regeneration of hepatocytes following LT (Alkozai et al. 2014). Direct evidence for this hypothesis is however not available.

Alkaline Phosphatase (ALP)

The enzyme ALP is responsible for dephosphorylation of multiple types of molecules. It is bound to plasma membrane lipoproteins of tissues throughout

the entire body. Serum ALP is mostly derived from liver parenchyma, biliary epithelium (cholangiocytes), and bone osteoblasts. To a lesser extent, serum ALP can also originate from intestinal mucosa, placenta, and kidney tissue (Kaplan 1972). The isoenzymes of intestinal and placental ALP are different from ALP in other tissues. It is possible to distinguish between the different isoenzymes, for instance, by elektropheresis. In clinical practice, however, ALP is generally tested together with GGT to differentiate. Strong elevation of both ALP and GGT indicates biliary obstruction, whereas extrahepatic obstruction causes a stronger rise in ALP compared to intrahepatic obstruction. Other hepatic causes for elevation consist of alcoholic abuse, hepatitis, and cholestatic disease. A sole elevation of ALP without rise in GGT levels indicates extrahepatic pathology, like bone disease or hyperthyroidism. In adults, serum values of ALP are $<$125 U/l. The halftime of most ALP isoenzymes is 3–7 days, while the halftime of intestinal ALP is $<$8 h.

Biomarkers to Assess Graft Function

Albumin

Albumin is one of the most abundant proteins in human serum and plasma besides blood coagulation factors. It is involved in pH homeostasis, maintaining oncotic pressure, and the transportation of blood compounds, hormones, and drugs. Synthesis takes place in the liver, and therefore, serum albumin is considered to be an important marker for liver function. Over 20 structural variants of albumin exist and its halftime is approximately 20 days. In healthy adults, serum/plasma levels are usually between 35 and 55 g/l, but levels can be influenced by body fluid distribution, for instance, by dehydration (Johnson 2006).

In particular hypoalbuminemia has been associated with liver disease and, following liver transplantation, with impaired graft function. A higher degree of graft injury, mirrored by high postoperative transaminase levels, often negatively affects liver graft function. However, the increased use of marginal grafts for liver transplantation has gained more interest for pure functional markers; because despite extensive injury, some marginal grafts manage to function well in recipients. Therefore, experimental studies with graft machine preservation focus on the assessment of liver function already prior to graft implantation in recipients (Bruinsma et al. 2014). But also following liver transplantation, early allograft dysfunction is estimated by a lack of markers that normally result from good liver function, like conjugated bilirubin and INR (coagulation). However, serum albumin is not included in this definition (Olthoff et al. 2010). Though albumin could be of use for assessing graft function, one should also be aware for other causes of hypoalbuminemia, like inflammation, malnutrition/malabsorption, malignancies, and hypothyroidism. Furthermore, albumin levels can remain in the normal range when patients suffer from biliary obstruction.

Bilirubin (Indirect and Direct)

Bilirubin is the yellow-colored breakdown product of hemoglobin when erythrocytes are degraded. A vast majority of bilirubin is derived from aged erythrocytes (over 85%), but ineffective erythropoiesis by bone marrow and certain hepatic enzymes can also contribute to bilirubin formation. When heme is degradated by splenic macrophages, unconjugated bilirubin is formed, which is not soluble in water and cannot be excreted. Subsequently, unconjugated bilirubin is bound to albumin and is transported to the liver, where hepatocytes conjugate bilirubin with glucuronic acid (90% diglucuronic, 10% monoglucuronic). This step makes bilirubin soluble in water and suitable for excretion via the hepatobiliary system. Once transported to the intestine and colon, conjugated bilirubin is hydrolyzed and reduced to urobilinogen by bacteria and excreted via the feces. A small part of the urobilinogen (2–5%) is resorbed into the enterohepatic circulation and excreted via the urine (Fevery 2008).

Human plasma or serum contains four fractions of bilirubin: unconjugated bilirubin (~27%), unconjugated bilirubin bound to albumin (~36%), monoconjugated bilirubin (~24%), and di-conjugated bilirubin (~13%). "Indirect" bilirubin consists of unconjugated bilirubin and the fraction of bilirubin not covalently bound to albumin. "Direct" bilirubin usually refers to fractions of conjugated bilirubin and bilirubin that is covalently bound to albumin. In clinical practice, total bilirubin and direct bilirubin are measurable in human serum or plasma. Total bilirubin consists of conjugated as well as unconjugated forms of bilirubin. Based on these measurements, the indirect bilirubin can be calculated with the formula: indirect bilirubin = total bilirubin – direct bilirubin. In healthy adults, total bilirubin levels are <20 μmol/l, and direct bilirubin levels are <5 μmol/l. Jaundice usually occurs when serum bilirubin exceeds 50 μmol/l (Marshall and Bangert 2005).

Based on total and direct bilirubin, one can distinguish different causes for hyperbilirubinemia. Strong elevation of unconjugated bilirubin indicates prehepatic pathophysiology like hemolysis or dysfunction of hepatocytes and conjugation at the hepatic level. However, most complications that can occur following liver transplantation will cause conjugated hyperbilirubinemia. At the hepatic level, hepatocyte injury due ischemia-reperfusion injury, EAD or PNF, is accompanied by a rise in direct bilirubin and liver transaminases. These changes can occur early after liver transplantation. In the case of intrahepatic cholestasis, for instance, due to biliary strictures, but also extrahepatic bile duct obstruction (post-hepatic level), hyperbilirubinemia is accompanied by a rise in ALP and GGT. Recurrence of (viral) hepatitis can elevate both conjugated and unconjugated serum bilirubin. Thus, by measuring conjugated and unconjugated hyperbilirubinemia and comparing serum levels with hepatocellular and cholestatic markers, one can distinguish between different complications following liver transplantation. When hepatocellular function is impaired, bilirubin levels also become measurable in urine and are per definition pathologic (Klatskin and Bungards 1953). When possible, collection of bile following liver transplantation can also be used for determining biliary bilirubin levels that can mirror hepatocyte function but also cholangiocyte injury (Verhoeven et al. 2015).

Prothrombin Time (PT) and International Normalized Ration (INR)

Synthesis of tissue factors for sufficient blood coagulation is an important function of the liver. A lack of tissue factors in blood plasma could indicate severe liver disease or, in the case of transplantation, graft failure. To assess the degree of graft failure or graft (dys)function following liver transplantation, one could measure individual coagulation factors, but instead, PT and INR are commonly used as general indicators.

Prothrombin time measures the time it takes for blood plasma to form a fibrin clot after adding tissue factor (III). In healthy individuals, PT is usually between 12 and 15 s but it depends on the standards of the laboratory performing the analysis. A prolonged PT could indicate a deficiency in the production of coagulation factors I (fibrinogen), II (prothrombin), V, VII, and X, which are all part of the extrinsic coagulation cascade. Logically, the use of anticoagulant drugs should be taken into account when interpreting PT. Immediately after liver transplantation, PT is usually prolonged and can reach up to 100 s. When PT does not decrease or normalize in the first postoperative week, this could indicate severe graft dysfunction with risk of developing serious complications and impaired patient survival. Urgent re-transplantation can be lifesaving in these cases. As mentioned before, the analysis and subsequent interpretation of PT is very institutionally dependent (Northup and Caldwell 2013).

Therefore, a standardized PT ratio, also known as the international normalized ratio (INR), is used more often to determine early allograft dysfunction. Outside the context of liver transplantation, INR is often used as a tool to monitor patients on vitamin K antagonists. The INR standardizes PT values of patients by calibrating reagents to an international sensitivity index (ISI) and by comparing patients' PT value with the mean PT of healthy individuals (normal), with the formula $INR = (PT_{patient}/PT_{normal})^{ISI}$(Kirkwood 1983). At 1 week following liver transplantation, INR is used as one of the parameters to evaluate early allograft dysfunction; an $INR \geq 1.6$ is considered to be a risk factor for shortened graft and recipient survival (Olthoff et al. 2010). Importantly, the cutoff of 1.6 seems to be a predictor of graft failure for grafts that were obtained from brain death donors as well as those obtained from circulatory death donors. Therefore, it has been suggested to give more weight to INR as a predictor of graft failure following liver transplantation (Croome et al. 2012).

Biomarkers for Recurrence of Disease Following Liver Transplantation

Besides the threat of cellular damage due to severe ischemia-reperfusion injury, biliary injury, and rejection, the recurrence of disease for which recipients were transplanted is also an important factor for graft loss. In particular PSC, HCC, and viral hepatitis B and C are notoriously recurring diseases in the transplanted graft (Kotlyar et al. 2006). Furthermore, over the last years, patients with unresectable cholangiocarcinoma are transplanted, but survival rates are limited due to recurrent or metastatic disease (Darwish Murad et al. 2012b). Several biomarkers are clinically

available to monitor recurrence of the abovementioned diseases in liver transplant recipients, which are described shortly in the following paragraphs.

Cholestatic Markers in Recurrence of PSC

Primary sclerosing cholangitis (PSC) is an autoimmune-related disorder that causes chronic inflammation and strictures of the (mainly intrahepatic) bile ducts. This progressive disease occurs more frequently in men compared to women and has been associated with ulcerative colitis (Lindor et al. 2015). Incidence is highest in the USA and north European countries. The time of onset until end-stage liver disease is approximately 12 years, and currently, LT is the only curative treatment for PSC. Unfortunately, recurrence of disease occurs in up to 20% of the PSC recipients, sometimes requiring re-transplantation (Hildebrand et al. 2015).

Clinical symptoms of recurrence of PSC consist of obstructive jaundice, bacterial cholangitis, fever, and fluctuating elevations of liver enzymes and cholestatic serum markers. Cholangiography shows typical intra- or extrahepatic strictures, beading, and irregularities. Histological features consist of fibrous cholangitis or fibro-obliterative lesions. Because of the overlap in clinical presentation with NAS, one of the criteria of recurrent PSC prescribes this diagnosis should be excluded if it develops within the first 90 days following LT (Graziadei et al. 1999). Besides recurrence, PSC patients also have an increased risk to develop CCA. Therefore, it could be plead to monitor these recipients for cancer antigen 19-9 (CA 19-9), a potential marker of CCA. Table 2 illustrates expected serum levels of classic biomarkers in PSC.

Cancer Antigen 19-9 (CA 19-9) in Recurrence of CCA

Cholangiocarcinoma is a rare disease that accounts for less than 3% of all gastrointestinal malignancies, but which has a poor prognosis due to its aggressive nature. Transplant centers recently started exploring the success of LT for perihilar CCA, either with or without the use of neoadjuvant chemo(radio)therapy (Darwish Murad et al. 2012a). In particular patients suffering from PSC have a 398-fold increased risk to develop CCA compared to the general population (Boonstra et al. 2013).

A potential serum marker to screen for (recurrent) CCA in PSC patients is CA 19–9. This carbohydrate structure is found in pancreatic tissue as well as on epithelial cells of the stomach and gallbladder. It can be secreted into serum by cancer cells. Besides cholangiocarcinoma, increased serum levels of CA 19-9 have been associated with pancreatic and colon cancer but also with benign causes of biliary obstruction. Therefore, when assessing the risk for a malignancy based on CA 19-9 serum levels, one should take into account whether cholestasis or cholangitis is present (preferring a cutoff value of $\geq$300 U/mL) or absent (better discrimination with a cutoff of $\geq$37 U/mL) (Kim et al. 1999). It is recommended to evaluate CA 19-9 levels after recovery of cholangitis. However, the optimal cutoff value for CA 19-9 remains inconclusive. A lower cutoff at 37 U/mL can be undesirable in terms of specificity, but higher cutoff values are at the

Table 2 Serum biomarkers during different pathophysiological states of the liver graft following LT. Values represent which serum levels can be expected for the various outcomes or diagnoses following LT. Except for PNF and EAD, these values are an indication and can diverge between different LT recipients

Complication following LT							
Biomarker	Healthy liver	PNF/EAD[a]	AS	NAS	ACR	Rec PSC	(Rec) HCC, CCA
AST (U/L)	<50	>2,000 within 7 days post-LT	=	50–400	100–1,000	50–400	=↑
ALT (U/L)	<50	>2,000 within 7 days post-LT	=	50–200	100–1,000	50–200	=↑
Total bili (μmol/L)	<20	≥170 on day 7 post-LT	= ↑	↑, up to 300	↑, up to 300	↑, up to 300	=↑
Albumin (g/L)	35–55	↓	=	=↓	↓	=	=↓
GGT (U/L)	<40	= ↑	↑ up to 500	↑, up to 200	↑, up to 300	↑, up to 200	=↑
ALP (U/L)	<125	= ↑	↑ up to 400	↑, up to 200	↑, up to 300	↑, up to 200	=↑
INR or PT (sec)	12–15	≥1.6 on day 7 post-LT	=	Prolonged	Prolonged	Prolonged	=↑
AFP (mcg/L)	<10–15	<10–15	<10–15	<10–15	<10–15	= ↑	↑
CA 19-9 (U/mL)	Neg	Neg	↑ prior to stenting	↑	Neg	If >100, higher risk of CCA	↑

[a]EAD (and PNF) are defined by serum biomarkers in the first week post-LT and consist of one of the following: serum AST or ALT > 2,000 U/L in the first postoperative week, total bilirubin levels ≥10 mg/dL (=170 μmol/L) on day 7 post-LT, or INR ≥1.6 on day 7 post-LT

Legend: Rec, recurrence = normal levels, ↑ increased levels, ↓decreased levels

expense of sensitivity (Levy et al. 2005). Current guidelines recommend a cutoff between 100 and 127 U/mL. Another important limitation of CA 19-9 is that its biosynthesis depends on the activity of fucosyltransferase-2 and fucosyltransferase-3 (FUT2 and FUT3, respectively). Individuals with inactive FUT3 do not express CA 19-9 on their epithelial cells. In contrast, FUT2 inactivity increases CA 19-9 expression. These genetic variations in FUT2 and FUT3 are not uncommon and strongly influence the optimal cutoff level for CA 19-9 in individuals (Wannhoff et al. 2013).

Finally, one could plea for use of CA 19-9 during follow-up after LT for cholangiocarcinoma, since posttransplant CA 19-9 levels are predictive of recurrence of cholangiocarcinoma (HR 1.8). This could influence the timing of adapted medical policy (Darwish Murad et al. 2012b).

Alpha-Fetoprotein (AFP) in Recurrence of HCC

The glycoprotein AFP is mainly produced in the fetal liver and yolk sac during gestation. In the first months after birth, plasma levels of AFP decrease and become undetectable at the age of approximately 1 year. In healthy adults, AFP levels are usually $<$10–15 μg/L (Tomasi 1977). Experimental animal studies have shown a role of AFP in estradiol transport and preventing virilization of female fetuses, but its function in humans remains largely unknown. After malignant degeneration, cells from various tissues are able to produce AFP. These cells can originate from the yolk sac, the gonads, hepatocytes, and certain gastric cells (Liu et al. 2010).

In patients with HCC, pre-transplant levels of AFP were shown to be predictive for recurrence of HCC during follow-up. Therefore, it has been suggested to incorporate pre-transplant AFP levels in the Milan criteria, which are currently used for screening of HCC patients to undergo LT (Duvoux et al. 2012). A rise in AFP levels during follow-up has also been associated with the recurrence of disease (Chaiteerakij et al. 2015; Macdonald et al. 2015). However, no clear correlation exists between AFP levels and tumor size, stage, or prognosis. Current guidelines advise to measure AFP every 3–6 months for 2 years combined with imaging in patients transplanted for HCC. After that, annual monitoring is sufficient. If AFP levels show a strong elevation, further diagnostics for possible recurrence should be undertaken.

Patients with chronic HBV or HCV infection have an increased risk to develop HCC. Serum levels of AFP can be elevated without the presence of an intrahepatic malignant process. However, AFP levels $>$500 mcg/L increase the risk of HCC (Wu 1990). Half-life of AFP is 5–7 days and is expected to decrease within 25–30 days after effective therapy.

Biomarker Dynamics in Various Complications Following LT

After discussing the specific markers for recurrent disease, the next paragraphs will provide an outline on biomarker dynamics that can be expected for common complications that can occur following LT.

Graft Primary Nonfunction (PNF) and Early Allograft Dysfunction (EAD)

Incidence of PNF is 5–8%, and despite being one of the most severe complications following LT, no formal definition of PNF exists. Usually, the diagnosis of PNF is ascertained by exclusion, and in retrospect, the transplanted liver fails to start functioning in the first postoperative days and requires liver re-transplantation or otherwise will inevitably result in the patients' death (Ploeg et al. 1993). Risk factors of PNF can be, for instance, donor related (high donor age, steatosis, small for size) or procedure related (prolonged cold or warm-ischemia times, donation after circulatory death, thrombosis (hepatic artery)) (Braat et al. 2012; Durand et al. 2008). However, in up to 50% of the cases, the exact cause of PNF remains unknown. Complete failure of the graft in PNF results in extremely elevated liver enzymes in serum, impaired or absent bile production, encephalopathy, and coagulopathy within the first 72 h following LT.

A complication similar to PNF is early allograft dysfunction (EAD). In 2010, Olthoff et al. formulated and validated criteria in order to determine EAD based on one or more of the following serum biomarker levels in the first week posttransplant: bilirubin $\geq$10 mg/dL on day 7, INR $\geq$1.6 on day 7, and ALT or AST levels $>$2,000 IU/L within the first 7 days. Though EAD is a risk factor for impaired graft and patient survival, in contrast to PNF, it will not inevitably result in liver re-transplantation or patient death. One could consider PNF as an excessive form of EAD, and therefore it might be questioned whether the two definitions should be fused. Furthermore, liver grafts obtained by donation after circulatory death (DCD) usually have poor immediate function and elevated serum biomarker levels, compared to donation after brain death (DBD). It has been suggested to adjust the definition of EAD for this category of LT in order to better assess the risk for graft failure (Croome et al. 2012). Especially since DCD is responsible for a significant contribution of the donor pool in many (particularly Western) countries, early prediction of EAD for this category could benefit graft and patient outcome. The median panel of Fig. 2 shows examples of biomarker dynamics during the first postoperative week in LT recipients suffering from PNF and EAD. Such dynamics are usually accompanied with extensive ischemic necrosis at the histological level.

Acute Cellular Rejection (ACR)

As explained before, the introduction of cyclosporine significantly improved graft survival by lowering the degree of cellular rejection. Nevertheless, in individual patients, it remains a challenge to lower immunosuppressant's use in order to avoid related complications, on one hand, and to prevent acute cellar rejection (ACR), on the other hand. ACR is the result of a T-cell-mediated immune response directed against tissue of the donor graft and mostly occurs within the first 90 days following LT (early ACR). However, low serum levels of immunosuppressant drugs have also been associated with ACR even years after transplantation (Mor et al. 1992). Clinical

symptoms in recipients consist of, fever, abdominal pain, hepatomegaly, and sometimes ascites. Laboratory test can show increased serum levels of hepatocellular and cholangiocyte-injury markers as well as bilirubin. The golden standard for diagnosing ACR however remains liver biopsy.

In 1995, experts formulated the so-called histological Banff criteria to evaluate the degree of ACR in liver biopsies, also known as the rejection activity index (Banff 1997). This index, outlined in Table 3, scores the extend of inflammation and lymphocytic infiltration into (i) the portal triads, (ii) the bile ducts, and (iii) the venous endothelium. To date, this index is used as part of standard clinical practice. In the early days, tissue biopsies were taken frequently post-LT to monitor for ACR but are now only indicated based on clinical symptoms.

Because of the low specificity of regular laboratory tests for ACR and the invasiveness of liver biopsies, many other surrogate biomarkers have been investigated to monitor for ACR, among which are interleukins, intercellular adhesion molecules, and many others. None have made it into clinical practice jet. A potential novel biomarker reported for ACR but also for other complications following LT is microRNAs (miRNAs), which will be discussed separately later.

Biliary Complications

Biliary complications are very common after liver transplantation and can vary in nature, location, and time of onset. The most common biliary complications consist of biliary leakage, anastomotic biliary strictures (AS), and non-anastomotic biliary strictures (NAS), which will all be discussed shortly.

Leakage of the biliary anastomosis usually occurs early following LT, and the cause is either technical or because of insufficient blood supply to the biliary tree resulting in biliary necrosis. Suspicion for biliary leakage rises when patients have pain and feel ill due to irritation of the peritoneum. Abdominal-free bile collections can be imaged by ultrasound but is more sensitive with ERCP, which is also useful for therapeutic stenting (Arain et al. 2013). Biliary leakage is often accompanied by AS.

Benign local narrowing or tapering at the site of the biliary anastomosis, also known as AS, occurs in approximately 5–10% of LT recipients. Shortly after LT, the biliary anastomosis can be edematous due to surgical trauma and/or ischemia. The development of AS does not depend on the type of biliary anastomosis (Verdonk et al. 2006). It is usually detected by elevated cholestatic markers in serum combined with clinical symptoms in recipients. Diagnosis and therapy of AS are accomplished by ERCP (Fig. 1a), and depending on the severity of the stricture, the bile duct can be cannulated by single or multiple stents (in the case of duct-duct) or by percutaneous drains (in the case of hepaticojejunostomy). If repeated attempts via the endoscopic or percutaneous route fail, AS can also be treated surgically (Balderramo et al. 2012). AS can occur early but also later following LT. Some recipients have recurrence of AS for which they need progressive stenting (Poley et al. 2013). Successful treatment of AS will result in a rapid decrease of cholestatic markers in serum and

Table 3 Banff scoring criteria or rejection activity index to evaluate histological graft rejection

Category	Description	Score
Portal inflammation	Mostly lymphocyte involving, but not noticeably expanding, a minority of the triads	1
	Expansion of most or all triads, by a mixed infiltrate containing lymphocytes with occasional blasts, neutrophils, and eosinophils	2
	Marked expansion of most or all triads by a mixed infiltrate containing numerous blasts and eosinophils with inflammatory spillover into the peripheral parenchyma	3
Bile duct inflammation/ damage	A minority of the ducts are cuffed and infiltrated by inflammatory cells and show only mild reactive changes such as increased nuclear-cytoplasmic ratio of the epithelial cells	1
	Most or all of the ducts are infiltrated by inflammatory cells. More than an occasional duct shows degenerative changes such as nuclear pleomorphism, disordered polarity, and cytoplasmic vacuolization of the epithelium	2
	As the above for two, with most or all of the ducts showing degenerative changes or focal luminal disruption	3
Venous endothelial inflammation	Subendothelial lymphocytic infiltration involving some, but not a majority, of the portal and/or hepatic venules	1
	Subendothelial infiltration involving most or all of the portal and/or hepatic venules	2
	Subendothelial infiltration involving most or all of the portal and/or hepatic venules as above for two, with moderate or severe perivenular inflammation that extends into the perivenular parenchyma and is associated with perivenular hepatocyte necrosis	3

patients can recover without residual symptoms. An example of cholestatic biomarker dynamics in AS is provided in the right panel of Fig. 2.

Besides the biliary anastomosis, some liver grafts develop strictures of the intrahepatic bile ducts or extrahepatic hilar region, which are called NAS (Buis et al. 2007). The method of postmortem donation strongly influences the risk for a liver graft to develop NAS: ~10% of DBD grafts versus ~30% of DCD grafts (Howell et al. 2012; O'Neill et al. 2014). Furthermore, it is known that thrombosis of the hepatic artery, the major supplier of blood to biliary tree, will inevitably lead to NAS. Therefore, warm ischemia is thought to play a key role in the pathophysiology of NAS. In contrast to AS, the (multiple) strictures in NAS and their anatomical localization are often less accessible for biliary stents or drains (Fig. 1b). Therefore, liver re-transplantation is indicated in 10–15% of all LT recipients due to NAS (Dubbeld et al. 2010). Large HAT usually indicates immediate liver re-transplantation. In serum, NAS give elevation of cholestatic markers, and only in few cases, normalization of biomarker levels to baseline is achieved. Eventually, NAS will lead to such severe cholestasis that patients will become ill and liver function will be affected.

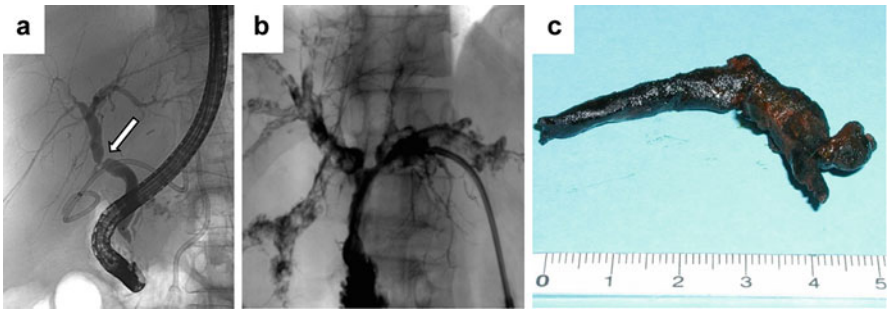

Fig. 1 Visualization of biliary complications following LT. (**a**) ERCP showing an isolated stricture at the biliary anastomosis, pointed out by the white arrow, with dilatation of the common bile duct and slim intrahepatic bile ducts. (**b**) ERCP showing dilated intrahepatic bile ducts throughout the entire liver graft with loss of normal architecture due to NAS. (**c**) Biliary cast removed from the hilar region of the liver graft that was formed due to obstruction and which is often seen in NAS. The length of the cast is displayed in cm. Pictures are derived from the database of the Erasmus Medical Center Rotterdam, The Netherlands

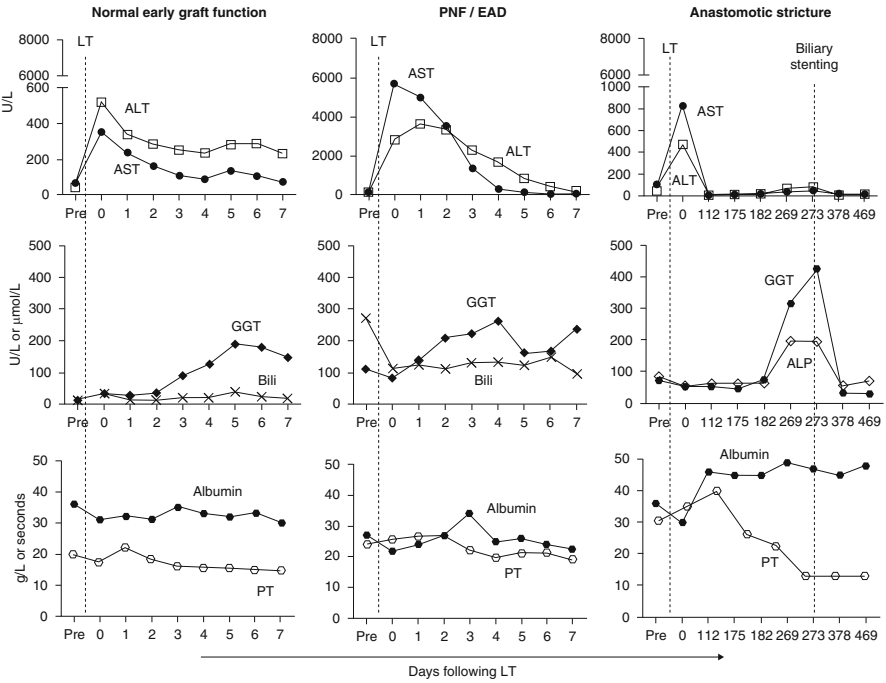

Fig. 2 Biomarker dynamics in blood and serum following LT. The *left* panels show biomarker dynamics in recipients with normal early graft function, the median panels show elevated biomarker levels in serum during PNF/EAD, and the *right* panels show increased cholestatic markers in AS. Data represent biomarker serum levels of individual patients following LT and were derived from the database of the Erasmus Medical Center Rotterdam, The Netherlands

Novel Biomarkers in the Field of Liver Transplantation

MicroRNAs (miRNAs) as Novel Biomarker

In the last decade, miRNAs have gained interest in the field of biomarker research. MicroRNAs are short, hairpin-shaped RNAs with the potential to regulate gene expression by inhibiting messenger RNA translation (Fig. 3). miRNAs are highly cell-type abundant and can be released via active and passive mechanisms into the circulation and other body fluids in which they remain stable up to 24 h. These characteristics make miRNAs attractive candidate biomarkers for various diseases. Besides their biomarker potential, the knowledge regarding miRNA-induced gene expression and regulation is increasing, though not yet fully understood (Farid et al. 2014).

For various liver diseases, particularly miR-122 has been related to hepatocellular liver injury. Serum levels of miR-122 increase earlier than conventional transaminase levels, which was shown in patients with viral hepatitis as well as in LT recipients who developed ACR (Farid et al. 2012; van der Meer et al. 2013). Therefore, hepatocyte-derived miRNAs (HDmiRs) might be suitable early markers for severe hepatocellular injury following LT, as is the case in grafts developing EAD or PNF. In contrast to liver transaminases, which are mainly injury markers, HDmiR-122 secretion into bile has also been correlated to good bilirubin excretion of hepatocytes into bile (Verhoeven et al. 2015). Therefore, HDmiR-122 and perhaps other HDmiRs might also be suitable markers for graft function.

Cholangiocytes have a different expression of miRNAs compared to hepatocytes (Chen et al. 2009). Therefore, cholangiocyte-derived miRNAs (CDmiRs) could be more sensitive or specific in the detection of biliary complications. Already at time of graft preservation, CDmiRs are released in response to ischemia-induced biliary injury that causes severe complications in LT recipients during follow-up (Verhoeven et al. 2013). Besides changes in expression, also the composition of miRNAs in bile is changed during biliary obstructions (Lankisch et al. 2014).

Despite the growing evidence of their utility, miRNAs as biomarker are currently not part of clinical practice in liver disease. Future research should focus on validation of sensitivity and specificity of previously identified CDmiRs and HDmiRs. Another challenge for implementing miRNAs as a routine laboratory test lies within the technical aspect of measuring miRNAs. This is now done by real-time quantitative polymerase-chain reaction (RT-qPCR), which takes approximately 3 h before miRNAs are isolated and analyzed. This issue could be facilitated by improving accelerated PCR techniques. Because of the highly sensitive analysis of qPCR, mild elevations of miRNA levels in blood or other body fluids can be determined quite accurately. Despite the fact that much is still unknown about miRNAs as therapeutic target, the first clinical series in human showed that inhibition of HDmiR-122 reduces viral load in HCV patients (Janssen et al. 2013). Whether CDmiRs are potentially interesting in (prevention of) cholestatic disease needs to be explored by future research.

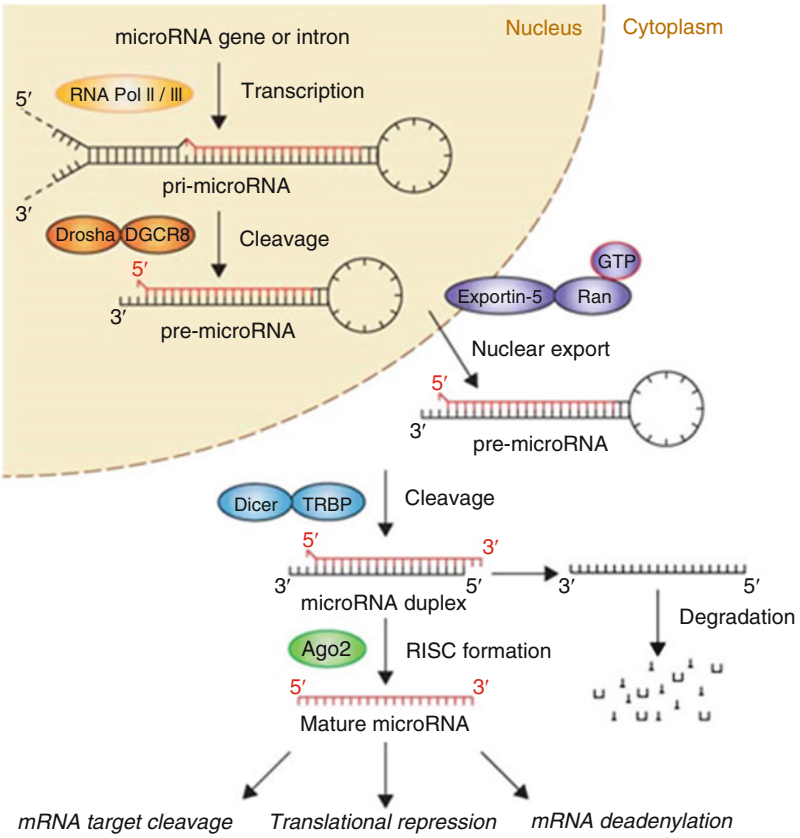

Fig. 3 MicroRNA structure and biogenesis. Biogenesis of miRNAs by cells. Immature precursor miRNAs are formed inside the cell nucleus. In the cell cytoplasma, miRNAs reach their mature form and are able to inhibit mRNA translation, thereby regulating gene expression. Illustration from (Winter et al. 2009)

Potential Application to Prognosis, Other Diseases, or Conditions

The previous paragraphs provided an overview of different types of biomarkers that are regularly used in liver disease and how these should be interpreted in the context of LT. Routine monitoring of graft quality based on biomarkers helps clinicians to decide whether or not to perform additional (mostly more invasive) tests like ERCP or liver biopsy. Biomarker levels can be the reason to adjust therapy, for instance, to increase immunosuppressant dosage when high transaminase levels indicate cellular rejection. But also as a definition of outcome, biomarkers play an important role in predicting prognosis early after LT.

Some important complications that can occur following LT, like EAD and biliary strictures, are often related to marginal quality of the liver graft already at time of

transplantation. As mentioned before, grafts obtained by DCD have a higher risk to develop EAD and NAS. For this reason, DCD liver grafts from elderly donors (over 60 years of age) are often rejected for LT. However, some of the rejected DCD grafts might have functioned well in recipients. With the increasing number of marginal grafts for LT, there is a need to improve and simultaneously to objectify graft quality in an earlier phase of LT. The prolonged time window between graft procurement and graft implantation, known as the preservation period, is in particular useful for this purpose. Many studies showed that during static cold storage, liver grafts can still release some injury markers that have been associated with outcome. A novel technique designed to preserve and improve graft quality is machine perfusion (MP) (Schlegel et al. 2015). With MP, the liver graft is flushed ex situ on a pump that recirculates preservation solution (perfusates) before implantation into the recipient. Many different techniques of MP have been investigated with variations in solutions, temperature, oxygenation, single-portal or dual portal-hepatic artery perfusion, flow pressure, and more. The first clinical studies with MP show promising results regarding prevention of hepatic and biliary injury (Dutkowski et al. 2015). However, during MP it remains a challenge to objectify that marginal grafts show enough recovery to be transplanted and which should still be rejected for LT. Multiple options are available to assess graft quality during MP with the use of biomarkers in graft perfusates and produced bile, depending on the applied technique (Verhoeven et al. 2014).

Despite the potential of biomarkers to assess graft quality during preservation, their clinical application is still experimental and the decision to accept a graft for LT is mainly driven by clinical donor variables and the macroscopic aspect on inspection by the donor surgeon. Besides donor variables, some researchers plea for the implementation of recipient variables as well in allocation algorithms, since recipient factors as age, MELD score, and gender can strongly influence survival (Blok et al. 2015). Because of the limited number of performed LTs annually in transplant centers, many biomarker studies omit validation of potential biomarkers in multiple cohorts. This will however delay the implementation of biomarkers to assess graft quality during preservation. Furthermore, criteria for EAD should be adapted for DCD liver grafts; despite their worse biomarker profile post-LT, multiple DCDs show good recovery during follow-up. The current criteria might be insufficient to distinguish grafts that will eventually function properly in recipients from the ones that actually cause PNF. This could also be the case for other types of donation, like living donor liver transplantation, for which another literature is recommended.

Summary Points and Discussion

To conclude, this overview discussed routinely measured biomarkers and more novel ones for evaluation of graft injury and function in the follow-up of LT recipients and their dynamics at time of various complications and (recurrence of) disease. It is evident that biomarkers can indicate hepatocellular injury, biliary obstruction, and liver function. Evaluation of biomarkers can play a key role in the

early recognition of complications and provide an objective tool to monitor graft quality after transplantation. As in recent years, many new potential biomarkers have been discovered; therefore this overview is incomplete and limited to established serum biomarkers. Furthermore, it should be emphasized that experienced clinical knowledge and imaging techniques of the liver are two other key factors in clinical decision making, and determining the need of intervention will rarely be based on biomarkers solely. Much likely, LT recipients will start with monitoring of graft function through biomarker measurements in the home situation as part of individualized medicine. Finally, novel application of biomarker measurements during graft preservation seems promising in the early evaluation of graft quality that could help extend the donor pool for LT.

References

Al-Hamoudi W, Elsiesy H, Bendahmash A, Al-Masri N, Ali S, Allam N, Al Sofayan M, Al Bahili H, Al Sebayel M, Broering D, Saab S, Abaalkhail F. Liver transplantation for hepatitis B virus: decreasing indication and changing trends. World J Gastroenterol. 2015;21(26):8140–7.

Alkozai EM, Lisman T, Porte RJ, Nijsten MW. Early elevated serum gamma glutamyl transpeptidase after liver transplantation is associated with better survival. F1000Res. 2014;3:85.

Arain MA, Attam R, Freeman ML. Advances in endoscopic management of biliary tract complications after liver transplantation. Liver Transpl. 2013;19(5):482–98.

Balderramo D, Sendino O, Burrel M, Real MI, Blasi A, Martinez-Palli G, Bordas JM, Carlos Garcia-Valdecasas J, Rimola A, Navasa M, Llach J, Cardenas A. Risk factors and outcomes of failed endoscopic retrograde cholangiopancreatography in liver transplant recipients with anastomotic biliary strictures: a case-control study. Liver Transpl. 2012;18(4):482–9.

Banff. Schema for grading liver allograft rejection: an international consensus document. Hepatology. 1997;25(3):658–63.

Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001;69(3):89–95.

Blok JJ, Putter H, Rogiers X, van Hoek B, Samuel U, Ringers J, Braat AE, Eurotransplant Liver Intestine Advisory C. The combined effect of donor and recipient risk on outcome after liver transplantation: research of the Eurotransplant database. Liver Transpl. 2015.

Boonstra K, Weersma RK, van Erpecum KJ, Rauws EA, Spanier BW, Poen AC, van Nieuwkerk KM, Drenth JP, Witteman BJ, Tuynman HA, Naber AH, Kingma PJ, van Buuren HR, van Hoek B, Vleggaar FP, van Geloven N, Beuers U, Ponsioen CY, Epi PSG. Population-based epidemiology, malignancy risk, and outcome of primary sclerosing cholangitis. Hepatology. 2013;58(6):2045–55.

Braat AE, Blok JJ, Putter H, Adam R, Burroughs AK, Rahmel AO, Porte RJ, Rogiers X, Ringers J, European, L., Intestine Transplant, A. & Eurotransplant Liver Intestine Advisory, C. The Eurotransplant donor risk index in liver transplantation: ET-DRI. Am J Transplant. 2012; 12(10):2789–96.

Bruinsma BG, Yeh H, Ozer S, Martins PN, Farmer A, Wu W, Saeidi N, Op den Dries S, Berendsen TA, Smith RN, Markmann JF, Porte RJ, Yarmush ML, Uygun K, Izamis ML. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am J Transplant. 2014;14(6):1400–9.

Buis CI, Verdonk RC, Van der Jagt EJ, van der Hilst CS, Slooff MJ, Haagsma EB, Porte RJ. Nonanastomotic biliary strictures after liver transplantation, part 1: radiological features and risk factors for early vs. late presentation. Liver Transpl. 2007;13(5):708–18.

Burroughs AK, Westaby D. Liver, biliary tract and pancreatic disease. In: Kumar, Clark (editors), Clin Med. 2005;349–52.

Calne RY, Rolles K, White DJ, Thiru S, Evans DB, McMaster P, Dunn DC, Craddock GN, Henderson RG, Aziz S, Lewis P. Cyclosporin A initially as the only immunosuppressant in 34 recipients of cadaveric organs: 32 kidneys, 2 pancreases, and 2 livers. Lancet. 1979;2(8151): 1033–6.

Cassidy WM, Reynolds TB. Serum lactic dehydrogenase in the differential diagnosis of acute hepatocellular injury. J Clin Gastroenterol. 1994;19(2):118–21.

Chaiteerakij R, Zhang X, Addissie BD, Mohamed EA, Harmsen WS, Theobald PJ, Peters BE, Balsanek JG, Ward MM, Giama NH, Moser CD, Oseini AM, Umeda N, Venkatesh S, Harnois DM, Charlton MR, Yamada H, Satomura S, Algeciras-Schimnich A, Snyder MR, Therneau TM, Roberts LR. Combinations of biomarkers and Milan criteria for predicting hepatocellular carcinoma recurrence after liver transplantation. Liver Transpl. 2015;21(5):599–606.

Chen L, Yan HX, Yang W, Hu L, Yu LX, Liu Q, Li L, Huang DD, Ding J, Shen F, Zhou WP, Wu MC, Wang HY. The role of microRNA expression pattern in human intrahepatic cholangiocarcinoma. J Hepatol. 2009;50(2):358–69.

Croome KP, Wall W, Quan D, Vangala S, McAlister V, Marotta P, Hernandez-Alejandro R. Evaluation of the updated definition of early allograft dysfunction in donation after brain death and donation after cardiac death liver allografts. Hepatobiliary Pancreat Dis Int. 2012;11(4):372–6.

Darwish Murad S, Kim WR, Harnois DM, Douglas DD, Burton J, Kulik LM, Botha JF, Mezrich JD, Chapman WC, Schwartz JJ, Hong JC, Emond JC, Jeon H, Rosen CB, Gores GJ, Heimbach JK. Efficacy of neoadjuvant chemoradiation, followed by liver transplantation, for perihilar cholangiocarcinoma at 12 US centers. Gastroenterology. 2012a;143(1):88–98. e3; quiz e14.

Darwish Murad S, Kim WR, Therneau T, Gores GJ, Rosen CB, Martenson JA, Alberts SR, Heimbach JK. Predictors of pretransplant dropout and posttransplant recurrence in patients with perihilar cholangiocarcinoma. Hepatology. 2012b;56(3):972–81.

De Ritis F, Coltorti M, Giusti G. An enzymic test for the diagnosis of viral hepatitis: the transaminase serum activities. 1957. Clin Chim Acta. 2006;369(2):148–52.

de Rooij BJ, van Hoek B, ten Hove WR, Roos A, Bouwman LH, Schaapherder AF, Porte RJ, Daha MR, van der Reijden JJ, Coenraad MJ, Ringers J, Baranski AG, Hepkema BG, Hommes DW, Verspaget HW. Lectin complement pathway gene profile of donor and recipient determine the risk of bacterial infections after orthotopic liver transplantation. Hepatology. 2010;52(3): 1100–10.

den Dulk AC, Sebib Korkmaz K, de Rooij BJ, Sutton ME, Braat AE, Inderson A, Dubbeld J, Verspaget HW, Porte RJ, van Hoek B. High peak alanine aminotransferase determines extra risk for nonanastomotic biliary strictures after liver transplantation with donation after circulatory death. Transpl Int. 2015;28(4):492–501.

Dubbeld J, Hoekstra H, Farid W, Ringers J, Porte RJ, Metselaar HJ, Baranski AG, Kazemier G, van den Berg AP, van Hoek B. Similar liver transplantation survival with selected cardiac death donors and brain death donors. Br J Surg. 2010;97(5):744–53.

Dueland S, Guren TK, Hagness M, Glimelius B, Line PD, Pfeiffer P, Foss A, Tveit KM. Chemotherapy or liver transplantation for nonresectable liver metastases from colorectal cancer? Ann Surg. 2015;261(5):956–60.

Durand F, Renz JF, Alkofer B, Burra P, Clavien PA, Porte RJ, Freeman RB, Belghiti J. Report of the Paris consensus meeting on expanded criteria donors in liver transplantation. Liver Transpl. 2008;14(12):1694–707.

Dutkowski P, Polak WG, Muiesan P, Schlegel A, Verhoeven CJ, Scalera I, DeOliveira ML, Kron P, Clavien PA. First comparison of hypothermic oxygenated perfusion versus static cold storage of human donation after cardiac death liver transplants: an international-matched case analysis. Ann Surg. 2015;262(5):764–71.

Duvoux C, Roudot-Thoraval F, Decaens T, Pessione F, Badran H, Piardi T, Francoz C, Compagnon P, Vanlemmens C, Dumortier J, Dharancy S, Gugenheim J, Bernard PH,

Adam R, Radenne S, Muscari F, Conti F, Hardwigsen J, Pageaux GP, Chazouilleres O, Salame E, Hilleret MN, Lebray P, Abergel A, Debette-Gratien M, Kluger MD, Mallat A, Azoulay D, Cherqui D, Liver Transplantation French Study, G. Liver transplantation for hepatocellular carcinoma: a model including alpha-fetoprotein improves the performance of Milan criteria. Gastroenterology. 2012;143(4):986–94. e3; quiz e14-5.

Eisenbach C, Encke J, Merle U, Gotthardt D, Weiss KH, Schneider L, Latanowicz S, Spiegel M, Engelmann G, Stremmel W, Buchler MW, Schmidt J, Weigand MA, Sauer P. An early increase in gamma glutamyltranspeptidase and low aspartate aminotransferase peak values are associated with superior outcomes after orthotopic liver transplantation. Transplant Proc. 2009;41(5):1727–30.

Farid WR, Pan Q, van der Meer AJ, de Ruiter PE, Ramakrishnaiah V, de Jonge J, Kwekkeboom J, Janssen HL, Metselaar HJ, Tilanus HW, Kazemier G, van der Laan LJ. Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. Liver Transplant. 2012;18(3):290–7.

Farid WR, Verhoeven CJ, de Jonge J, Metselaar HJ, Kazemier G, van der Laan LJ. The ins and outs of microRNAs as biomarkers in liver disease and transplantation. Transpl Int. 2014;27(12): 1222–32.

Fevery J. Bilirubin in clinical practice: a review. Liver Int. 2008;28(5):592–605.

Giannini EG, Testa R, Savarino V. Liver enzyme alteration: a guide for clinicians. CMAJ. 2005; 172(3):367–79.

Goldberg DM. Structural, functional, and clinical aspects of gamma-glutamyltransferase. CRC Crit Rev Clin Lab Sci. 1980;12(1):1–58.

Graziadei IW, Wiesner RH, Batts KP, Marotta PJ, LaRusso NF, Porayko MK, Hay JE, Gores GJ, Charlton MR, Ludwig J, Poterucha JJ, Steers JL, Krom RA. Recurrence of primary sclerosing cholangitis following liver transplantation. Hepatology. 1999;29(4):1050–6.

Hildebrand T, Pannicke N, Dechene A, Gotthardt DN, Kirchner G, Reiter FP, Sterneck M, Herzer K, Lenzen H, Rupp C, Barg-Hock H, de Leuw P, Teufel A, Zimmer V, Lammert F, Sarrazin C, Spengler U, Rust C, Manns MP, Strassburg CP, Schramm C, Weismuller TJ, German P. S. C. S. G. Biliary strictures and recurrence after liver transplantation for primary sclerosing cholangitis – a retrospective multicenter analysis. Liver Transpl. 2015 [Epub ahead of print].

Hooijkaas H, Mohrmann K, Smeets LC, Souverijn JHM, Tax GHM. Aspartaataminotransferase, *Handboek medische laboratoriumdiagnostiek*. Houten: Prelum; 2013a. p. 123–6.

Hooijkaas H, Mohrmann K, Smeets LC, Souverijn JHM, Tax GHM. Lactaatdehydrogenase, *Handboek medische laboratoriumdiagnostiek*. Houten: Prelum; 2013b. p. 485–6.

Howell JA, Gow PJ, Angus PW, Jones RM, Wang BZ, Bailey M, Fink MA. Early-onset versus late-onset nonanastomotic biliary strictures post liver transplantation: risk factors reflect different pathogenesis. Transpl Int. 2012;25(7):765–75.

Huang CF, Yeh ML, Tsai PC, Hsieh MH, Yang HL, Hsieh MY, Yang JF, Lin ZY, Chen SC, Wang LY, Dai CY, Huang JF, Chuang WL, Yu ML. Baseline gamma-glutamyl transferase levels strongly correlate with hepatocellular carcinoma development in non-cirrhotic patients with successful hepatitis C virus eradication. J Hepatol. 2014;61(1):67–74.

Jain A, Reyes J, Kashyap R, Dodson SF, Demetris AJ, Ruppert K, Abu-Elmagd K, Marsh W, Madariaga J, Mazariegos G, Geller D, Bonham CA, Gayowski T, Cacciarelli T, Fontes P, Starzl TE, Fung JJ. Long-term survival after liver transplantation in 4,000 consecutive patients at a single center. Ann Surg. 2000;232(4):490–500.

Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, van der Meer AJ, Patick AK, Chen A, Zhou Y, Persson R, King BD, Kauppinen S, Levin AA, Hodges MR. Treatment of HCV infection by targeting microRNA. N Engl J Med. 2013;368(18):1685–94.

Johnson AM. Proteins. In: Burtis CA, Ashwood ER, Bruns DE, editors. Tietz textbook of clinical chemistry and molecular diagnostics. 3rd ed. Philadelphia: Saunders; 2006. p. 546–9.

Kaplan MM. Alkaline phosphatase. N Engl J Med. 1972;286(4):200–2.

Kim HJ, Kim MH, Myung SJ, Lim BC, Park ET, Yoo KS, Seo DW, Lee SK, Min YI. A new strategy for the application of CA19-9 in the differentiation of pancreaticobiliary cancer: analysis using a receiver operating characteristic curve. Am J Gastroenterol. 1999;94(7):1941–6.

Kirkwood TB. Calibration of reference thromboplastins and standardisation of the prothrombin time ratio. Thromb Haemost. 1983;49(3):238–44.
Kirsch JF, Eichele G, Ford GC, Vincent MG, Jansonius JN, Gehring H, Christen P. Mechanism of action of aspartate aminotransferase proposed on the basis of its spatial structure. J Mol Biol. 1984;174(3):497–525.
Kishi Y, Abdalla EK, Chun YS, Zorzi D, Madoff DC, Wallace MJ, Curley SA, Vauthey JN. Three hundred and one consecutive extended right hepatectomies: evaluation of outcome based on systematic liver volumetry. Ann Surg. 2009;250(4):540–8.
Klatskin G, Bungards L. An improved test for bilirubin in urine. N Engl J Med. 1953;248(17): 712–7.
Kotlyar DS, Campbell MS, Reddy KR. Recurrence of diseases following orthotopic liver transplantation. Am J Gastroenterol. 2006;101(6):1370–8.
Lankisch TO, Voigtlander T, Manns MP, Holzmann A, Dangwal S, Thum T. MicroRNAs in the bile of patients with biliary strictures after liver transplantation. Liver Transpl. 2014;20(6):673–8.
Levy C, Lymp J, Angulo P, Gores GJ, Larusso N, Lindor KD. The value of serum CA 19-9 in predicting cholangiocarcinomas in patients with primary sclerosing cholangitis. Dig Dis Sci. 2005;50(9):1734–40.
Lindor KD, Kowdley KV, Harrison ME, American College of, G. ACG clinical guideline: primary sclerosing cholangitis. Am J Gastroenterol. 2015;110(5):646–59. quiz 660.
Liu X, Cheng Y, Sheng W, Lu H, Xu Y, Long Z, Zhu H, Wang Y. Clinicopathologic features and prognostic factors in alpha-fetoprotein-producing gastric cancers: analysis of 104 cases. J Surg Oncol. 2010;102(3):249–55.
Lum G, Gambino SR. Serum gamma-glutamyl transpeptidase activity as an indicator of disease of liver, pancreas, or bone. Clin Chem. 1972;18(4):358–62.
Macdonald B, Sewell JL, Harper AM, Roberts JP, Yao FY. Liver transplantation for hepatocellular carcinoma: analysis of factors predicting outcome in 1074 patients in OPTN Region 5. Clin Transplant. 2015;29(6):506–12.
Marshall WJ, Bangert SK. Clinical Chemistry. Philadelphia: Saunders; 2005. p. 375.
Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med. 1996;334(11):693–9.
Mor E, Gonwa TA, Husberg BS, Goldstein RM, Klintmalm GB. Late-onset acute rejection in orthotopic liver transplantation – associated risk factors and outcome. Transplantation. 1992; 54(5):821–4.
Northup PG, Caldwell SH. Coagulation in liver disease: a guide for the clinician. Clin Gastroenterol Hepatol. 2013;11(9):1064–74.
O'Neill S, Roebuck A, Khoo E, Wigmore SJ, Harrison EM. A meta-analysis and meta-regression of outcomes including biliary complications in donation after cardiac death liver transplantation. Transpl Int. 2014;27(11):1159–74.
Olthoff KM, Kulik L, Samstein B, Kaminski M, Abecassis M, Emond J, Shaked A, Christie JD. Validation of a current definition of early allograft dysfunction in liver transplant recipients and analysis of risk factors. Liver Transpl. 2010;16(8):943–9.
op den Dries S, Buis CI, Adelmeijer J, Van der Jagt EJ, Haagsma EB, Lisman T, Porte RJ. The combination of primary sclerosing cholangitis and CCR5-Delta32 in recipients is strongly associated with the development of nonanastomotic biliary strictures after liver transplantation. Liver Int. 2011;31(8):1102–9.
Op den Dries S, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw AS, Leuvenink HG, Lisman T, Porte RJ. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PLoS One. 2014;9(2): e88521.
Ploeg RJ, D'Alessandro AM, Knechtle SJ, Stegall MD, Pirsch JD, Hoffmann RM, Sasaki T, Sollinger HW, Belzer FO, Kalayoglu M. Risk factors for primary dysfunction after liver transplantation – a multivariate analysis. Transplantation. 1993;55(4):807–13.

Poley JW, Lekkerkerker MN, Metselaar HJ, Kuipers EJ, Bruno MJ. Clinical outcome of progressive stenting in patients with anastomotic strictures after orthotopic liver transplantation. Endoscopy. 2013;45(7):567–70.

Schlegel A, Kron P, Dutkowski P. Hypothermic oxygenated liver perfusion: basic mechanisms and clinical application. Curr Transplant Rep. 2015;2(1):52–62.

Starzl TE, Marchioro TL, Vonkaulla KN, Hermann G, Brittain RS, Waddell WR. Homotransplantation of the liver in humans. Surg Gynecol Obstet. 1963;117:659–76.

Starzl TE, Groth CG, Brettschneider L, Moon JB, Fulginiti VA, Cotton EK, Porter KA. Extended survival in 3 cases of orthotopic homotransplantation of the human liver. Surgery. 1968;63(4): 549–63.

Strimbu K, Tavel JA. What are biomarkers? Curr Opin HIV AIDS. 2010;5(6):463–6.

Tomasi Jr TB. Structure and function of alpha-fetoprotein. Annu Rev Med. 1977;28:453–65.

van der Meer AJ, Farid WR, Sonneveld MJ, de Ruiter PE, Boonstra A, van Vuuren AJ, Verheij J, Hansen BE, de Knegt RJ, van der Laan LJ, Janssen HL. Sensitive detection of hepatocellular injury in chronic hepatitis C patients with circulating hepatocyte-derived microRNA-122. J Viral Hepat. 2013;20(3):158–66.

Verdonk RC, Buis CI, Porte RJ, van der Jagt EJ, Limburg AJ, van den Berg AP, Slooff MJ, Peeters PM, de Jong KP, Kleibeuker JH, Haagsma EB. Anastomotic biliary strictures after liver transplantation: causes and consequences. Liver Transpl. 2006;12(5):726–35.

Verhoeven CJ, Farid WR, de Ruiter PE, Hansen BE, Roest HP, de Jonge J, Kwekkeboom J, Metselaar HJ, Tilanus HW, Kazemier G, van der Laan LJ. MicroRNA profiles in graft preservation solution are predictive of ischemic-type biliary lesions after liver transplantation. J Hepatol. 2013;59(6):1231–8.

Verhoeven CJ, Farid WR, de Jonge J, Metselaar HJ, Kazemier G, van der Laan LJ. Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation. J Hepatol. 2014;61(3):672–84.

Verhoeven CJ, Farid WR, Roest HP, Ramakrishnaiah V, de Ruiter PE, de Jonge J, Kwekkeboom J, Metselaar HJ, Tilanus HW, Kazemier G, Ijzermans JN, van der Laan LJ. Polarized release of hepatic microRNAs into bile and serum in response to cellular injury and impaired liver function. Liver Int. 2015 [Epub ahead of print].

Wannhoff A, Hov JR, Folseraas T, Rupp C, Friedrich K, Anmarkrud JA, Weiss KH, Sauer P, Schirmacher P, Boberg KM, Stremmel W, Karlsen TH, Gotthardt DN. FUT2 and FUT3 genotype determines CA19-9 cut-off values for detection of cholangiocarcinoma in patients with primary sclerosing cholangitis. J Hepatol. 2013;59(6):1278–84.

WHO. International programme on chemical safety. Biomarkers in risk assessment: validity and validation. 2001. Available online: http://www.inchem.org/documents/ehc/ehc/ehc222.htm

Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol. 2009;11(3):228–34.

Wroblewski F. The clinical significance of alterations in transaminase activities of serum and other body fluids. Adv Clin Chem. 1958;1(2):313–51.

Wu JT. Serum alpha-fetoprotein and its lectin reactivity in liver diseases: a review. Ann Clin Lab Sci. 1990;20(2):98–105.

Biomarkers of Extracellular Matrix Remodeling in Liver Diseases

10

Mette J. Nielsen, Diana J. Leeming, Morten A. Karsdal, and Aleksander Krag

Contents

M.J. Nielsen (✉) • D.J. Leeming (✉) • M.A. Karsdal (✉)
Nordic Bioscience, Biomarkers and Research, Herlev, Denmark
e-mail: mju@nordicbioscience.com; mette.juni@gmail.com; djl@nordicbioscience.com; mk@nordicbioscience.com

A. Krag
Department of Gastroenterology and Hepatology, University of Southern Denmark, Odense University Hospital, Odense C, Denmark
e-mail: aleksander.krag@rsyd.dk

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_14

Abstract

The common denominator, regardless of the underlying etiology, of chronic liver diseases is liver fibrosis. The hallmark of fibrosis is the accelerated accumulation of extracellular matrix proteins, ultimately leading to loss of organ function. The extracellular matrix consists of fibrous and nonfibrous macromolecules such as collagens, elastin, and proteoglycans.

During progressive fibrosis the balance of extracellular matrix remodeling is altered and leads to changes in quality, quantity, and distribution of extracellular matrix proteins in the liver. This results in excessive accumulation of fibrous tissue and an overall change in protein profile and liver structure and increase in extracellular matrix density. A cirrhotic liver may contain up to six times as much collagen as a healthy liver with type I and III collagen as the most abundant ones. The imbalanced extracellular matrix remodeling leads to a range of formation and disease-relevant degradation products of extracellular and intracellular proteins into the circulation. These fragments may provide information about the pathogenesis of disease and serve as serological biomarker targets. Clinically there is a need to identify early stages of liver fibrosis and particularly to detect patients with fast progressing fibrosis to prevent further progression.

In contrast to liver biopsy, serological markers are believed to reflect both the activity of the fibrotic processes as well as the total extracellular matrix mass undergoing remodeling. This chapter discusses the most common extracellular matrix remodeling markers such as hyaluronic acid, N-terminal procollagen type III, and type IV collagen as well as a novel alternative, called the protein fingerprint technology. This technology has received increased attention due to the diagnostic and prognostic potentials as serological biomarkers of extracellular matrix remodeling in various diseases, including chronic liver diseases.

Keywords

Extracellular matrix • Remodeling • Fibrosis • Collagen • Biomarkers • Neoepitope

List of Abbreviations

ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUC	Area under receiver operator characteristics curve
BIPED	Burden of disease, investigative, prognostic, efficacy of intervention, diagnostic
C3M	Type III collagen degradation fragment
ECM	Extracellular matrix
ELF	Enhanced liver fibrosis
FACIT	Fibril-associated collagens
FDA	Food and drug administration
HA	Hyaluronic acid

HALT-C	Hepatitis C antiviral long-term treatment against cirrhosis
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HSC	Hepatic stellate cells
MMP	Matrix metalloproteinase
NAFLD	Nonalcoholic liver disease
NPV	Negative predictive value
PIIINP	N-terminal type III collagen propeptide
PPV	Positive predictive value
Pro-C3	True type III collagen formation fragment
PTM	Posttranslational modification
TIMP	Tissue inhibitor of metalloproteinase

Key Facts of ECM Remodeling Biomarkers

- Around 45% of all deaths are associated with some kind of fibroproliferative disease, characterized by altered extracellular matrix remodeling.
- The gold standard for diagnosis of liver fibrosis is by histopathological evaluation of liver biopsies, a highly invasive procedure with many drawbacks such as patient discomfort and sampling error.
- One of the most pressing needs in clinical chemistry is the development of better biomarkers for early diagnosis, prognosis, and early efficacy of treatment for better personalized heath care and improved drug development.
- The perfect noninvasive marker of liver fibrosis should be liver specific, easy to perform, reproducible, accurate, and inexpensive. In addition, it should both stage liver fibrosis and also monitor disease progression and treatment efficacy. However, no such marker exists.
- An extensive search for noninvasive alternatives has been ongoing for the past decades, resulting in a long list of serological or imaging-based biomarkers.
- Generally, serological biomarkers of liver fibrosis are classified as either Class I, i.e., direct biomarkers reflecting extracellular matrix turnover and the changes in the fibrogenic cell type (however, they do not indicate the extent of extracellular matrix protein distribution), or Class II, i.e., the indirect biomarkers mostly estimate the degree of fibrosis.
- The extracellular matrix is a supramolecular structure of protein aggregates generating a dynamic scaffold in which cells are linked together in a three-dimensional network controlling cell-matrix interactions and cell fate during up- or downregulation of proteases. The extracellular matrix consists of fibrous and nonfibrous macromolecules, i.e., collagens, elastin, laminins, and fibronectin, as well as proteoglycans and other glycoproteins, respectively.
- Neo-epitope biomarkers are based on a unique combination of specific protein and its cleavage of a disease-specific protease, thus representing the end product of tissue destruction.

- Neo-epitope biomarkers may more accurately quantify the effects of converging pathways leading to fibrosis, thus consequently be related to diagnosis, prognosis, and efficacy of intervention.

Definitions of Words and Terms

Collagen	The most abundant family of proteins found in connective tissue, such as the skin, bone, tendons, liver, kidney, lung, etc. The collagens have a characteristic structure of three α-chains supercoiled around each other in a right-handed triple helix. The helix contains a glycine at every third amino acid, forming a characteristic – glycine-X-Y repeat – where X- and Y-positions are frequently proline and 4-hydroxyproline.
Collagenase	A group of enzymes specific for proteolytic processing of collagens.
Cytokine	A protein secreted by cells, which can modify the behavior of other cells.
Extracellular matrix	The macromolecular ground substance of connective tissue consisting of proteins, proteoglycans, and polysaccharides linking cells together in a three-dimensional framework.
Extracellular matrix remodeling	Removal and replacement of old or damaged extracellular matrix components with new, intact ones. The remodeling can either be controlled physiologic remodeling or uncontrolled pathologic remodeling.
Fibrosis	Excessive extracellular matrix deposition as a consequence of sustained wound healing response.
Glycoprotein	A group of proteins containing carbohydrates in the form of monosaccharide units attached to specific amino acid residues.
Neo-epitope	A new epitope on an antigen generated by disease-specific protease cleavage which can be recognized by an antibody.
Polysaccharide	A class of high-molecular-weight carbohydrates linked together by condensation of monosaccharides or their derivatives forming linear or branched chains.

Protein fingerprint	A unique combination of a protein and its disease-specific protease.
Serology	The use of antibodies to quantify antigens.

Introduction

Approximately 45% of all deaths in the Western world are associated with some kind of chronic fibroproliferative disease. Therefore the need and marked potential for anti-fibrotic drugs that are both safe and effective are huge. Consequently there is an increasing focus among researchers, funding agencies, and the pharmaceutical industry to develop effective anti-fibrotic therapies. Chronic liver disease is often asymptomatic in early stages and manifests at advanced stages of cirrhosis; thus there is a clinical need of earlier disease identification, of better treatment and prevention of complications to cirrhosis, and to identify fast progressors.

An important challenge in the development of reliable and robust biomarkers is the heterogeneity of chronic liver diseases. The fibrotic process is not linear and may change during disease course. Therefore some patients will be fast or slow progressors, while others may not advance in fibrosis grade. Thus the management and follow-up of these patients should be differentiated dependent on the rate of fibrosis progression. At present no tools are available for reliable monitoring of fibrosis and/or progression of this; thus there is a clear need for new and better biomarkers (Schuppan and Kim 2013).

In recent years there has been an extensive search for novel markers for the noninvasive evaluation of liver fibrosis. The development of novel markers is based on two distinct but complementary approaches: a biological approached based on surrogate markers such as serological or urinary biomarkers of liver fibrosis and a physical approach based on imaging measurement of liver stiffness. Despite the limitations of liver biopsy, histological evaluation of the liver tissue remains the standard against which novel noninvasive methods are benchmarked. With the variability in interpreting biopsy results, the technique may be considered a non-perfect gold standard, and therefore the perfect noninvasive diagnostic marker would not be perfectly correlated to liver biopsy findings. This means that if a liver biopsy was 90% specific and sensitive, the perfect biomarker could only obtain an accuracy of maximum 0.90 (Mehta et al. 2009).

The perfect noninvasive marker of liver fibrosis should be liver specific, easy to perform, reproducible, accurate, and inexpensive. In addition, it should both be able to stage liver fibrosis and monitor disease progression and treatment efficacy (Manning and Afdhal 2008). Better noninvasive biomarkers may provide patients and their physicians with a better and earlier assessment of diagnosis and progression as well as surveillance of progression and response to curative and preventive strategies as outlined in Table 1.

Table 1 Potential benefit of biochemical markers of fibroproliferative diseases for patients and health-care systems. The table highlights the clinical unmet need, patient benefits, as well as the cost-effectiveness of biochemical markers as diagnostic and prognostic tests (The table is modified from Karsdal et al. (2014) with permission

Biochemical markers of fibrosis	Clinical unmet need	Patient benefit	Cost-effectiveness
Diagnostic test	Tests that are easy to obtain and can be applied in any clinical setting to enable:	Noninvasive (based on blood sample)	Cheaper than invasive tests
	Diagnosis of patients at any given stage of fibrosis	Early diagnosis of asymptomatic disease	Initiation of treatment at a later stage
	Correct staging of fibrosis (mild/moderate/severe) by a simple blood test	Time saving	Optimal management of patients
		Differentiation of diagnosis resulting in personalized health care	
Prognostic test	Tests that can be applied repeatedly in any clinical setting to assess:	Identification of a threshold for treatment	Rational and more focused clinical decision making
	Risk of progression toward poorer prognosis	Identification of patients at high-risk organ failure	Identification of those patients in most need of treatment
	Identification of patients in need of treatment	Personalized follow-up strategy	Better and cheaper clinical decision making
	Identification of patients whose treatment could be deferred		

Extracellular Matrix

The extracellular matrix (ECM) is a common denominator in most fibroproliferative disorders, as fibrosis is defined as a consequence of sustained wound healing and accelerated accumulation of ECM proteins. The ECM constitutes a substantial part of all mammalian tissues. The ECM is a supramolecular structure with the ability to form protein aggregates. This results in a dynamic scaffold linking cells together in a three-dimensional network controlling cell-matrix interactions and cell fate during up- or downregulation of proteases. ECM consists of fibrous and nonfibrous macromolecules, i.e., collagens, elastin, laminins, and fibronectin, as well as proteoglycans and other glycoproteins, respectively. The various amounts and combinations of the different proteins form a unique and balanced scaffold with specific functions and needs for the individual tissue.

The ECM is subdivided into the interstitial matrix and the basement membrane, each having diverse roles depending on the organ and location of the tissues

(Bruckner 2010). The interstitial matrix makes up most of the ECM in the body. It consists of different types of collagens, fibronectin, and proteoglycans. The collagens provide tensile strength and hydraulic conductivity properties, while proteoglycans control cell adhesion and have osmotic pressure properties, all of which contributes to tissue homeostasis, and exchange of nutrients and waste products (Frantz et al. 2010; Heinegard 2009). The basement membrane is a specialized ECM which controls various biological processes such as cell adhesion, migration and development, tissue regeneration, and wound healing and serves as a reservoir of growth factors and enzymes. The most widespread proteins of the basement membrane are type IV collagen, which is responsible for the mechanical stability of the basement membrane, as well as laminin, perlecan, and entactin/nidogen (Yurchenco and Schittny 1990).

Remodeling of the ECM is an important physiologic process of growth, repair, and development of tissues. These processes are being controlled by specific ECM-modifying enzymes that regulate degradation and de novo synthesis of ECM components. The balance between synthesis and degradation must be tightly controlled in order to maintain tissue integrity (Fig. 1). However, if the remodeling balance is disturbed, it may lead to an altered matrix composition either with increased synthesis or increased degradation of the ECM. In case of continuous imbalanced remodeling as seen during pathological processes, the altered matrix composition, i.e., fibrosis, may ultimately result in loss of tissue function (Karsdal et al. 2013).

In addition to quantitative changes in ECM protein distribution, there is also a change in protein quality caused by posttranslational modifications (PTMs) during

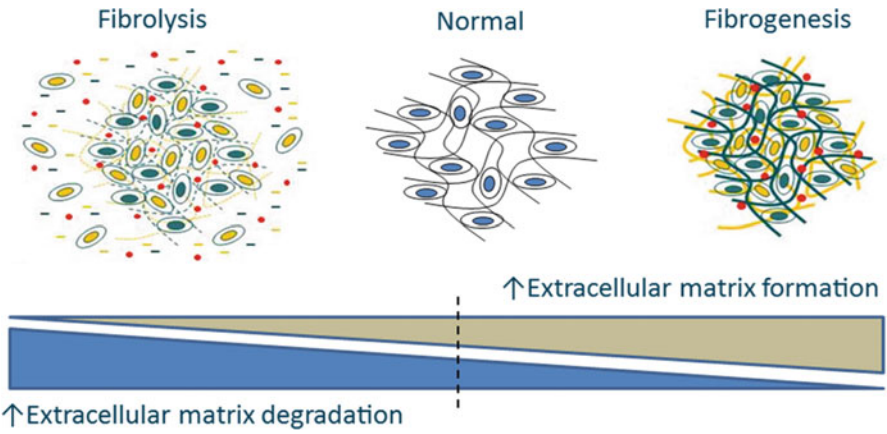

Fig. 1 Extracellular matrix quantity versus quality. Under normal physiologic tissue homeostasis, the ratio of extracellular matrix formation and degradation is well balanced. During pathological conditions, the balance is disturbed leading to more extracellular matrix degradation and less formation (fibrinolysis) (*left*) or more extracellular matrix formation and less degradation (fibrogenesis) (*right*) (The figure has been modified from Karsdal et al. (2015) with permission)

pathological conditions. PTMs are non-DNA-coded modifications to the protein after translation, which amplify the structural and functional diversity of the protein. PTMs of proteins embedded in the ECM are of great importance for generating stable ECM structures and are suggested to be consequences of both physiologic and pathologic conditions. Depending on the underlying condition, several PTMs have been identified, including amino acid isomerization in aging, protease degradation in inflammation and fibrosis, citrullination in inflammation, and glycosylation in diabetes (Karsdal et al. 2010). In fibrotic tissues, the lack of ECM degradation is caused by transglutaminases which mediates cross-linking of ECM preventing proteolytic cleavages of collagens. This ultimately results in increased number and accumulation of collagens and disorganized tissue (Grenard et al. 2001).

Collagen

The main type of protein in the ECM is the collagen family, which consists of 28 different types encoded by more than 40 different genes. The collagens have a characteristic structure consisting three α-chains, either identical (homotrimers) or nonidentical (heterotrimers), which are supercoiled around each other in a right-handed triple helix. The helix contains a glycine at every third amino acid, forming a characteristic glycine-X-Y repeat, where X- and Y-positions are frequently occupied by proline and 4-hydroxyproline limiting rotation of the polypeptide chain. The repeat organizes the glycines to face the center, while amino acids with longer side chains face outward, allowing a closer packaging along the central axis in the triple helix. The triple helix is further stabilized by hydrogen bonds and water bridges (Fig. 2). The collagens are classified according to their subfamilies and supramolecular organization, including

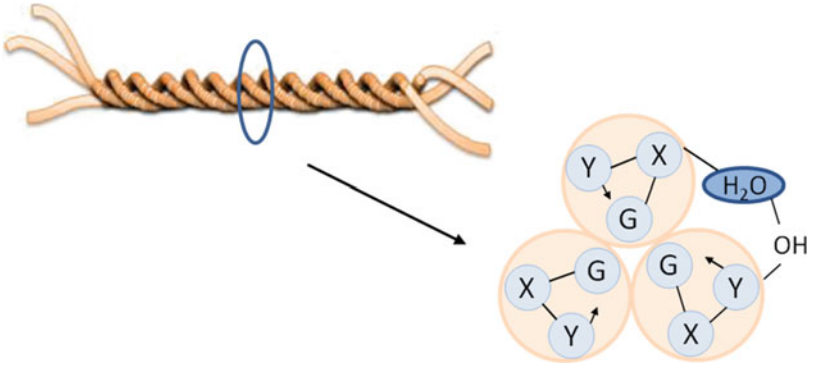

Fig. 2 Collagen structure. The tertiary structure of the collagen molecule consists of three intertwined α-chains forming a triple helix. The glycines (G) face the center to allow for a closer packaging of the helix. X- and Y-positions are frequently occupied by proline and 4-hydroxyproline. Hydrogen bonds (OH) and water bridges (H_2O) further stabilize the helix. The figure is made by authors from literature description

fibril-forming collagen, basement membrane collagens, network-forming collagens, fibril-associated collagens (FACIT), anchoring fibrils, transmembrane collagens, and other collagens with unique functions (Ricard-Blum and Ruggiero 2005).

Collagens in the Normal Liver

The ECM of the liver only constitutes around 3% of the relative area in a normal liver section and is mainly restricted around Glisson's capsule, portal tracts, sinusoid walls, and central veins. More than 20 different types of collagens have been identified in the liver. The most abundant collagens of the liver are the fibrillar type I, III, and V collagen, the basement membrane type IV and XVIII collagen, microfibrillar type VI collagen, and small amount of the FACIT type XIV collagen. The fibrillar collagens are located around the portal tract and central vein walls, while type IV collagen along with non-fibrillar proteins, laminin and entactin/nidogen, forms a low-density basement membrane-like matrix along the sinusoid walls. This allows for an easy diffusion between blood and liver cells as well as maintaining differentiated functions of surrounding hepatocytes and sinusoidal cells (Bedossa and Paradis 2003; Schuppan et al. 2001).

Fibrillar collagens are synthesized as procollagen molecules each flanked by two non-collagenous domains called the N- and C-terminal propeptides. When the procollagen is secreted from the cell, the propeptides are cleaved off by specific proteases leaving the mature collagen able to assemble into fibrils through the N- and C-terminal telopeptides, a non-collagenous domain located between the propeptide and the major triple helical domain (Exposito et al. 2010).

Type IV collagen is exclusively found in the basement membrane and differs in structure from the fibrillar collagens. Type IV collagen consists of six homologue α-chains, α1(IV)-α6(IV). Each chain consists of three distinct domains: the N-terminal 7S domain, the central triple helical part, and a C-terminal non-collagenous domain (NC1). The chains assemble into three heterotrimers with tissue-specific distributions. The predominant form is presented by $[\alpha1(IV)]_2\alpha2$ (IV) forming the basement membrane network in most embryonic and adult tissues including the liver (Ricard-Blum and Ruggiero 2005). The type IV collagen network serves as binding site for other ECM molecules such as laminin, perlecan, and proteoglycans, thereby providing the basis for specialized tissue-specific basement membranes (Badylak et al. 2009).

Type VI collagen is a heterotrimer of three different α-chains with short triple helical domains and extended globular termini. Two type VI collagen monomers associate in an antiparallel fashion upon secretion into the ECM. Subsequently two dimers form tetramers that further associate end-to-end to form a microfibrillar network. Type VI collagen is localized as part of the interstitial matrix of the liver displaying a prominent pericellular localization. It has multiple interactions with other ECM proteins, growth factors, matrix metalloproteinases (MMPs), and cell surface receptors. Due to these interactions, type VI collagen is believed to play an important role in tissue homeostasis (Schuppan et al. 2001).

MMPs and Their Inhibitors

So far, four different types of proteolytic enzymes capable of degradation of ECM have been identified. These include the MMPs, serine proteases, cysteine proteases, and aspartic proteinases. The MMPs are considered to be essential for collagen degradation, although some of the other proteolytic enzymes might also be able to degrade collagens, such as cathepsins belonging to the cysteine protease group.

MMPs are a family of zinc-dependent endopeptidases. Twenty-five different MMPs have been identified in vertebrates and they are grouped according to their domain structure. All MMPs have the same core structure with a signal peptide, propeptide with a cysteine switch, a catalytic domain containing three zinc-binding histidines, a linker peptide region of various lengths, and a hemopexin domain. Based on the composition of the domains and the preferred substrate, the MMP family is subdivided into five groups: collagenases, gelatinases, stromelysins, matrilysins, and transmembrane-type MMPs (Fanjul-Fernandez et al. 2010).

It is generally believed that the collagenases (MMP-1, MMP-8, and MMP-13) are responsible for the initial cleavage of the collagens in two characteristic ¼ and ¾ fragments after which the collagens are prone to further degradation by the gelatinases (MMP-2 and MMP-9). For effective collagenolytic activity, the enzyme must be able to bind to collagen, unwind the triple helix, and cleave the individual strands of the collagen (Chung et al. 2004). The preferred substrate for the collagenases is interstitial type I, II, and III collagen, but they are also able to degrade other ECM molecules and soluble proteins. The gelatinases prefer, as the name imply, gelatins, i.e., partly degraded collagen, and are therefore able to degrade a wide range of ECM proteins, including types I, II, III, IV, VI, VII, IX, and X, elastin, fibronectin, aggrecan, vitronectin, and laminin (Overall 2002). Furthermore, the gelatins are also responsible for the degradation of non-ECM molecules, and they release several pro- or angiogenic factors (Egeblad and Werb 2002).

The MMPs not only play a major role in various cellular and physiological events such as cell migration and tissue remodeling but also in pathological conditions such as inflammation, fibrosis, and cancer (Hu et al. 2007). The regulation of MMP activity is complex involving tight regulation at several levels. The activity is regulated by two types of endogenous inhibitors, namely, α2-macroglobulin and tissue-inhibitors of metalloproteinases (TIMPs). Human α2-macroglobulin is able to inhibit most proteinases by entrapment of the proteinase within the macroglobulin followed by a rapid clearance by receptor-mediated endocytosis. TIMPs are a family of inhibitors binding in a 1:1 stoichiometry to the active catalytic site of MMPs. Four different types of TIMPs have been identified in vertebrates, all of which play an important role in physiological tissue remodeling as well as under pathological conditions where the changes in TIMP levels are considered to be direct reflectors of the MMP activity level (Visse and Nagase 2003).

ECM Components and Their Distribution During Liver Fibrosis

During fibrosis progression the quantity and quality of the hepatic ECM change with an up to fivefold increase in total collagen content along with a change in the collagen profile resulting in twice the amount of type I collagen compared to type III collagen (Schuppan 1990). The increase in collagen deposition is subsequently followed by a shift in matrix composition from the low-density basement membrane-like matrix to an interstitial matrix containing fibril-forming collagens (Schuppan et al. 2001) (Fig. 3).

Regardless of etiology, cirrhosis is the end stage of progressive fibrogenesis; however the developmental pattern of fibrosis depends on the underlying etiology (Cassiman and Roskams 2002). Hepatic stellate cells (HSCs) have been considered the main fibrogenic cell type, possibly due to its ability to become isolated from human and rodent liver tissue. Therefore most of the knowledge related to hepatic fibrosis has been based on in vitro activation of HSC. However, several fibroblast-like cell types contributing to fibrosis development have been identified, including septal and interface myofibroblasts and smooth muscle cells (bdel-Aziz et al. 1991; Friedman 1993; Gressner 1994).

In chronic viral hepatitis, the fibrotic pattern develops as portal-central septa as a consequence of portal-central bridging necrosis with initial histological changes characterized by inflammatory cell infiltration and matrix deposition around portal tracts (Ramadori and Saile 2004). In alcoholic and metabolic liver diseases, the fibrotic pattern resembles a "chicken wire" in which the fibrillar matrix is deposited around groups of hepatocytes and sinusoids (Cassiman and Roskams 2002). In biliary fibrosis the portal fibroblasts undergo rapid activation upon injury resulting in increased expression of α-smooth muscle actin and secretion of type I collagen. This initially results in periportal fibrosis subsequently followed by portal-portal septa formation surrounding the liver nodules. The central vein and its connections to the portal tracts are preserved until later stages where HSCs are main contributors to disease progression (Knittel et al. 1999).

Biomarkers of Liver Fibrosis

Generally, serological biomarkers of liver fibrosis are classified as either Class I, i.e., direct biomarkers reflecting ECM turnover and the changes in the fibrogenic cell type (however, they do not indicate the extent of ECM protein distribution), or Class II, i.e., the indirect biomarkers mostly estimate the degree of fibrosis. The two biomarker classes follow different pathophysiological concepts, in which direct markers reflect what is going on, i.e., the grade of fibrogenic activity, while indirect markers reflect where fibrosis is deposited, i.e., stage of fibrosis (Gressner and Gao 2014).

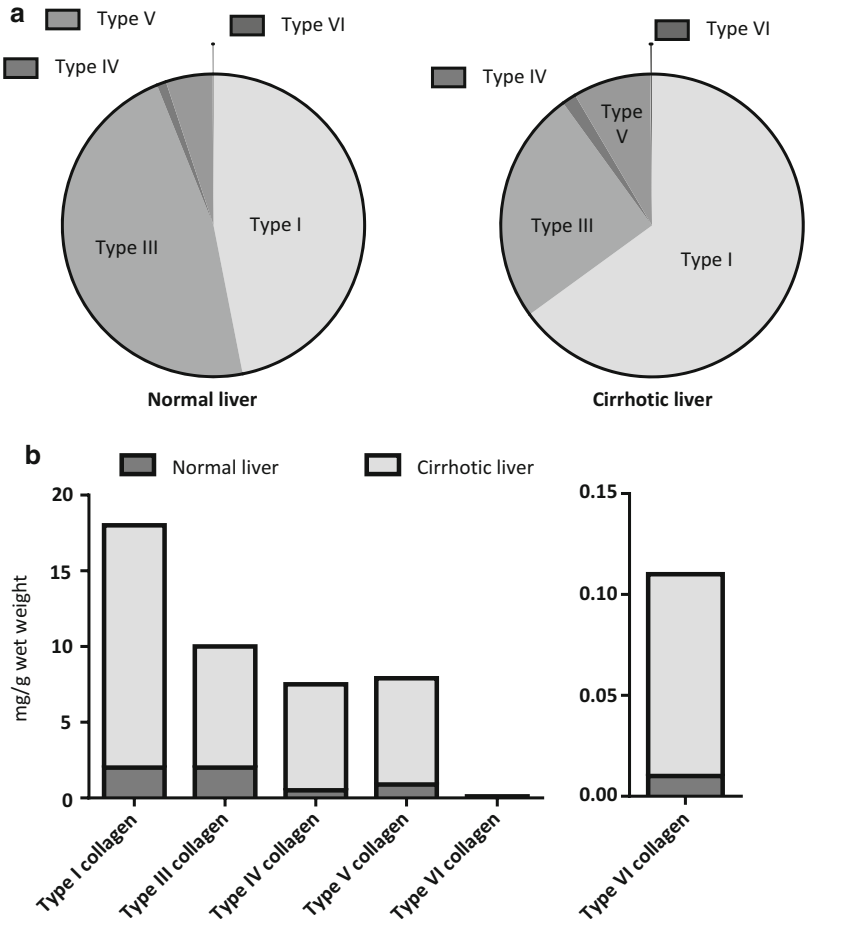

Fig. 3 Comparison of collagen content between normal and cirrhotic liver. (**a**) Percentage of total collagen quantity in normal and cirrhotic livers. Type I, IV, V, and VI collagen increases from 40–50% to 60–70%, 1% to 1–2%, 2–5% to 5–10%, and 0.1% to 0.2%, respectively, while type III collagen decreases from 40–50% to 20–30% in cirrhotic liver compared to normal liver. (**b**) Wet weight increase of collagen. The wet weight of type I, III, IV, V, and VI collagen increases 8, 4, 14, 8, and 10 times, respectively, in cirrhotic liver compared to normal liver. The figure is made by authors by data accumulation from literature

Class I: Direct

Direct markers of liver fibrosis include cytokines and markers of matrix metabolism and are summarized in Table 2. As the ECM turnover involves new ECM deposition and removal as well as remodeling of established ECM, direct markers are believed to reflect both the activity of the fibrotic processes as well as the total ECM mass undergoing remodeling. This is based on the following three findings: (1) the

Table 2 Examples of Class I liver fibrosis biomarkers. The table summarizes present Class I fibrosis biomarkers of extracellular matrix remodeling with their clinical and disease-specific application

Class I (direct markers) with clinical application				
Marker	Method	Clinical application	Disease application	Sensitivity/ specificity (%)
HA	RIA, ELISA	+	ALD, viral	86/88
PIIINP	RIA	+	ALD, viral	78/81
YKL-40	RIA/ELISA	(+)	ALD	
Type IV collagen 7S	RIA	(+)	Viral	
Type IV collagen NC1	RIA/ELISA	(+)	Viral	
MMP-2	ELISA	(+)	HCV	
TIMP-1 and TIMP-2	ELISA	(+)	HCV	
Laminin	RIA, ELISA	(+)	ALD	
PINP	ELISA	–		
PICP	RIA	–		
Tenascin	ELISA	–		
ELF test	ADVIA Centaur (PIIINP, TIMP-1, HA)	+	Mixed	90/41
Leroy score	RIA (PIIINP), ELISA (MMP-1)	+	HCV	60/92

The table has been modified from Gressner and Gao (2014) with permission
ALD alcoholic liver disease, *EIA* enzyme immunoassay, *ELF test* enhanced liver fibrosis test, *ELISA* enzyme-linked immunosorbent assay, *HA* hyaluronic acid, *MMP* matrix metalloproteinase, *PIN/CP* N/C-terminal propeptide of type I collagen, *RIA* radioimmunoassay, *TIMP* tissue inhibitor of metalloproteinases, *YKL-40* human cartilage glycoprotein 39

markers are often more elevated in conditions with rapid fibrosis progression (Ramadori et al. 1991), (2) serum levels of the markers decrease in response to treatment of the underlying disease (Yamada et al. 1996), and (3) most of the markers correlate with the stage of fibrosis rather than biochemical or histological features of inflammation (Leroy et al. 2001).

The common markers of ECM remodeling can further be subdivided according to their molecular structure, i.e., collagens, glycoproteins and polysaccharides, collagenases and their inhibitors, and cytokines. The best known collagen markers include the propeptides of type I and III collagen (PINP and PIIINP), reflecting interstitial collagen formation, as well as type IV collagen, reflecting basement membrane remodeling (Koivisto et al. 2007; Leroy et al. 2001; Murawaki et al. 2001).

The glycoprotein biomarkers include hyaluronic acid (HA), laminins, tenascin, and YKL-40, all of which are associated with the basement membrane. HA is one of the few serological markers which have reached clinical application, since it is able to exclude advanced fibrosis and cirrhosis; however it has not yet obtained general acceptance (Guechot et al. 1996). Laminin is responsible for organizing the basement membrane, and the marker has shown to be increased in patients with fibrosis

and to correlate very well with the degree of portal hypertension (El-Mezayen et al. 2015). YKL-40 is a fibroblast growth factor which may be involved in ECM remodeling as increased level of YKL-40 has been found in patients with liver fibrosis (Johansen et al. 2000).

Collagenases and their inhibitors include MMPs and TIMPs, especially MMP-2 and TIMP-1 and TIMP-2. These proteins are involved in degradation of ECM and to permit new matrix deposition. Clinical studies show contradictory results showing that MMP-2 is related to fibrosis in some studies, while no clinical relevance is found in other studies (Boeker et al. 2002; Walsh et al. 1999).

Cytokines have also been studied as potential biomarkers of liver fibrosis as they are involved in the modulation of ECM cross talk. The best known cytokine as biomarker for liver fibrosis is TGF-β, while other potential cytokine markers include TNF-α and interleukins (Radwan et al. 2012).

Diagnostic Direct Markers of Liver Fibrosis

General for all the direct markers is the lack of assay standardization as well as variation in histological definitions and patient populations. This makes it difficult to draw strong conclusions about which biomarker performs best. In addition, the changes in biomarker level also seem to be affected by the underlying disease or pattern of fibrosis deposition as some markers appear to have a good diagnostic value while others do not (Afdhal and Nunes 2004). Furthermore, the serum levels of these markers are altered by changes in clearance, metabolism, and secretion. For instance, clearance of HA in the circulation is dependent upon binding to specific receptors on hepatic endothelial cell surface, and thus the increase in HA in the postprandial state suggests a competition for the receptors (Idobe et al. 1998).

Several clinical studies have evaluated the diagnostic utility of direct markers in comparative studies. Many of these find HA superior to PIIINP for the diagnosis of cirrhosis (Guechot et al. 1996; Murawaki et al. 1995; Oberti et al. 1997). For example, in patients with alcoholic liver disease (ALD), HA had a good diagnostic accuracy of 0.86, while some of the other including PIIINP and laminin were less impressive with diagnostic accuracies of 0.74 and 0.81, respectively (Oberti et al. 1997). In patients with hepatitis C virus infection (HCV), HA has also shown its superiority over PIIINP both for diagnosis of fibrosis and cirrhosis with nearly perfect diagnostic accuracies around 0.90 for HA compared to 0.70 for PIIINP (Guechot et al. 1996). The differences in diagnostic accuracies might be explained by the differences in the correlation of HA and PIIINP histological staging and markers of inflammation (Murawaki et al. 1995).

Several assays for the assessment of type IV collagen have been developed and seem to perform similar to each other. Type IV collagen has in many studies been assessed together with HA; however it seems like there is no clear advantage of using the type IV collagen assays over HA for staging fibrosis. In some studies the type IV collagen assays appear to have a slightly better diagnostic potential than HA (Murawaki et al. 1995, 1996), while other studies find that HA performs better than

type IV collagen (Marinho et al. 2010; Xie et al. 2003). In one study, the investigators assessed two different assays targeting either the triple helix or the 7S domain of type IV collagen and found that the diagnostic accuracy for identifying cirrhosis in patients with viral hepatitis was better for the 7S assay (Murawaki et al. 1996).

TIMP-1 has mostly been investigated as a marker of liver fibrosis in combination with HA and PIIINP, known as the enhanced liver fibrosis (ELF) test. Serum levels of TIMP-1 have shown to be highly upregulated in patients with HCV and seem to be correlated with the histological degree of fibrosis (Boeker et al. 2002). In a study investigating TIMP-1 as diagnostic marker of chronic HBV infection, the investigators found the diagnostic value of TIMP-1 to be higher than the diagnostic values of PIIINP, HA, and type IV collagen (Zhu et al. 2012). The use of MMP-2 as noninvasive marker of liver fibrosis has shown mixed results, in which some studies find a strong correlation to fibrosis stages (Murawaki et al. 2001) and others find a weak correlation (Walsh et al. 1999), whereas some even find no correlation at all (Kasahara et al. 1997). One of the difficulties in obtaining repeated reliable results for enzyme biomarkers might be explained by that the circulating level active MMPs or TIMPs is limited. One might speculate that these enzymes responsible for the degradation of the ECM in the body are in their inactive state when circulating; thus targeting the active form of proteases and their inhibitors might not be possible.

YKL-40 is expressed in areas of active fibrogenesis as growth factor of fibroblasts and endothelial cells (Johansen et al. 1997). Serum levels of YKL-40 have shown to be elevated in patients with various etiologies of liver fibrosis, especially alcoholic cirrhosis (Johansen et al. 2000; Tran et al. 2000). Furthermore YKL-40 correlated to the degree of fibrosis with the highest levels found in patients with moderate to severe fibrosis (Johansen et al. 2000).

Protein Fingerprint: A Novel Approach in Direct Biomarker Development

The hallmark of liver fibrosis is imbalanced ECM remodeling. The process results generation of degradation and formation products of extracellular and intracellular proteins generated by disease-specific proteases expressed at the site of injury and release into circulation. The unique combination of a protein and its protease constitutes a protein fingerprint of the ongoing changes in a specific tissue and may provide information about the pathogenesis of disease and serve as biomarker target (Karsdal et al. 2010, 2011) (Fig. 4).

The protein fingerprint technology enables measurement of sub-pools of the same protein, as these fragments may provide different information emphasizing the importance of distinguishing each sub-pool from others. An example of this is the measurement of type I collagen in which three different markers measure three sub-pools of this protein: (1) CTX-I detects aged bone resorption as it measures an isomerized, cross-linked neo-epitope degraded by cathepsin K; (2) P1NP detects bone formation as

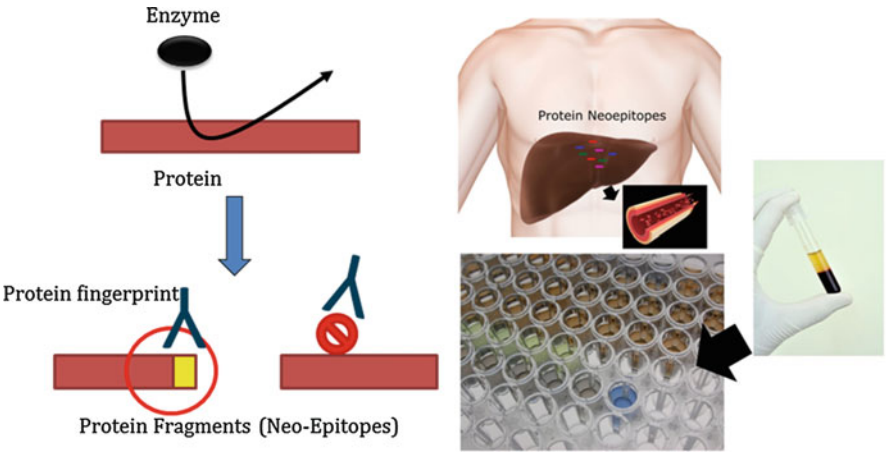

Fig. 4 Protein fingerprint technology. During protease degradation of structural proteins, the protein fragments expose unique ends (neo-epitopes) (*left*). Specific antibodies can be raised against the newly exposed ends. The neo-epitope fragment is unique for a combination of a protease and a structural protein and so is the antibody. The protein fragments will be released into the circulation (*right*) and captured in a blood sample and may be used in the assessment of individual diseases. The figure is made by authors

it targets the propeptide, which is cleaved off during incorporation of the parent molecule into the ECM; and (3) C1M detects soft tissue turnover as it targets a neo-epitope generated by MMP and destroyed by cathepsin K (Karsdal et al. 2011).

The neo-epitope approach has received increased attention due to the diagnostic and prognostic potentials as serological biomarkers of ECM remodeling in bone and cartilage diseases (Dam et al. 2009; Reijman et al. 2004; Schaller et al. 2005). As the neo-epitope biomarkers represent the turnover products of the fibrotic structure, this type of markers may consequently be related to diagnosis, prognosis, and efficacy of intervention (Karsdal et al. 2010) and has in recent years been implemented in fibrotic diseases in the lungs and liver (Jenkins et al. 2015; Leeming et al. 2013b, 2014).

Table 3 describes the current serological biomarkers based on the protein fingerprint technology. Many of the markers have been validated in a CCl_4 rat model as biomarkers of ECM remodeling of liver fibrosis. One study included nine biomarkers, i.e., degradation markers of type I, III, IV, and VI collagen, citrullinated vimentin, and biglycan, as well as formation markers of type III, IV, and V collagen. The study found that all nine markers were related to the extent of liver fibrosis (Leeming et al. 2013a). As the markers can be used for translational science, the markers have later been validated in patients with chronic HCV infection, alcoholic cirrhosis, and HIV (Jansen et al. 2014; Leeming et al. 2013b, 2014, 2015; Nielsen et al. 2015a). Regardless of the cause of fibrosis, the studies concluded that the serum levels of all markers increased along with increased severity of either fibrosis or degree of portal hypertension; thus the novel markers can be used as a tool to monitor ECM remodeling in CLD.

Table 3 Currently available serological protein fingerprint markers for the assessment of extracellular matrix structure. The table shows currently available protein fingerprint markers to assess either extracellular matrix formation or degradation. Each biomarker reflects a specific neo-epitope generated by the combination of an extracellular or intracellular matrix protein and a protease

Type	Marker	Protein	Protease
Formation	P1NP	Type I collagen	–
	Pro-C3	Type III collagen	ADAMTS
	P4NP7S	Type IV collagen 7S	–
	Pro-C5	Type V collagen	BMP-1/ADAMTS
	Pro-C6	Type VI collagen	BMP-1
Degradation	C1M	Type I collagen	MMP
	C2M	Type II collagen	MMP
	C3M	Type III collagen	MMP
	C4M	Type IV collagen	MMP
	C5M	Type V collagen	MMP
	C6M	Type VI collagen	MMP
	BGM	Biglycan	MMP
	CRPM	C-reactive protein	MMP
	ELM	Elastin	MMP
	EL-NE	Elastin	HNE
	MIM	Mimecan	MMP
	TIM	Titin	MMP
	VCANM	Versican	MMP
	VICM	Vimentin	MMP
	CTX-I	Type I collagen	Cat K
	CTX-II	Type II collagen	MMP

ADAMTS A disintegrin and metalloproteinase with thrombospondin motifs, *BMP-1* bone morphogenic protein 1, *Cat K* cathepsin K, *HNE* human neutrophil elastase, *MMP* matrix metalloproteinase. The table has been made by authors by literature data collection

In most fibroproliferative diseases, several different pathways often contribute to the development of the disease. Therefore, quantification of a single factor in a highly complex process may not reflect the nature of the disease due to redundancy. Serological neo-epitope biomarkers represent the end product of tissue destruction and may more accurately quantify the effects of converging pathways.

Class II: Indirect

Indirect markers of liver fibrosis generally include the common clinical chemistry tests, such as enzymes, proteins, platelets, and coagulation factors, which not necessarily reflect ECM turnover or fibrogenic cell changes. Their pathobiochemical relation with fibrogenesis is indirect, hence the name. Thus the indirect markers are out of the scope for this chapter and will only be introduced briefly.

Table 4 Examples of Class II liver fibrosis biomarkers. The table summarizes present Class II fibrosis biomarkers based on algorithms showing the included parameters in each test, as well as their disease application

Class II (indirect markers)			
Marker	Parameters	Disease application	Sensitivity/ specificity (%)
FibroTest	Haptoglobin, α2-macroglobulin, GGT, apolipoprotein A1, bilirubin	HBV, HCV	75/85
Actitest	FibroTest + ALT	HCV	89/75
APRI	AST, platelet count	HCV	89/75
Hepascore	Bilirubin, γ-GT, HA, α2-macroglobulin, age, gender	HCV	63/89
FIB-4	Platelet count, AST, ALT, age	HCV/HIV	70/74
Forns index	Age, platelet count, GGT, cholesterol	HCV	94/51
Fibrometer	Platelet count, prothrombin index, AST, α2-macroglobulin, HA, urea, age	Mixed	81/84
Pohl score	AST/ALT-ratio, platelet count	HCV	41/99
Patel index	HA, TIMP-1, α2-macroglobulin	HCV	77/73
Sud index	Age, AST, cholesterol, insulin resistance, past alcohol intake	HCV	96/44
Testa index	Platelet count/spleen diameter ratio	HCV	78/79
PGA index	Prothrombin time, GGT, apolipoprotein A1	Mixed	91/81
PGAA index	Prothrombin time, GGT, apolipoprotein A1, α2-macroglobulin	ALD	79/89

The table has been modified from Gressner and Gao (2014) with permission
ALD alcoholic liver disease, *ALT* alanine aminotransferase, *AST* aspartate amino transferase, *GGT* γ-glutamyltransferase, *HA* hyaluronic acid, *HCV* hepatitis C virus, *HIV* human immunodeficiency virus, *TIMP* tissue inhibitor of metalloproteinases

The number of biomarkers in this class is rapidly increasing with a great variety of biochemical scores and biomarker panels based on statistical models and mathematical algorithms (Table 4). The most prevalent tests are the FibroTest™ (and ActiTest™ for necroinflammatory activity), APRI score, Hepascore, and the ELF test. The ELF test is currently the first fibrosis score which is further developed as routine medical test (Gressner and Gao 2014).

The European Liver Fibrosis Study was the first international, multicenter, cross-sectional study with the aim of identifying an optimal panel of serological ECM markers combined in an algorithm to estimate the severity of liver fibrosis (Rosenberg et al. 2004). Rosenberg et al. combined PIIINP, TIMP-1, and HA along with age in a logistic regression model and tested the performance of the algorithm in different liver diseases. The algorithm was able to detect significant fibrosis and cirrhosis with sensitivities above 90% for HCV, ALD, and nonalcoholic fatty liver disease (NAFLD). The performance of the ELF test in

predicting clinical outcomes was evaluated with a 7-year follow-up cohort of patients with various causes of chronic liver diseases. The study found that the ELF test was at least as accurate as liver biopsies in predicting liver-related outcomes given a one-point increase in ELF increased the risk of a liver-related outcome twofold (Parkes et al. 2010).

Most of the markers are able to diagnose extreme stages of fibrosis, i.e., no/limited fibrosis versus advanced fibrosis/cirrhosis. However diagnosing the "gray zone" between these two extremes still remains a clinical challenge (Pinzani 2010). The lack of accuracy can be explained by the combination of individually assessed parameters, which creates relatively high variance due to limited analytical imprecision and unstandardized methods (Gressner et al. 2009). Thus the use of multi-marker panels has to be taken with caution in clinical practice.

Potential Applications to Prognosis, Other Diseases, or Conditions

Prognostic Direct Markers of Liver Fibrosis

Another important characteristic of a liver fibrosis marker is its utility as prognostic marker regarding progression of liver fibrosis, predicting clinical outcomes, or assessing the efficacy of treatments. Unfortunately, the available data is limited. The largest prospective study of chronic hepatitis C progression is the Hepatitis C Antiviral Long-term Treatment Against Cirrhosis (HALT-C) Trial. The trial was designed to investigate maintenance and clinical outcomes in patients who failed to eradicate HCV during prior interferon therapy (Lee et al. 2004). In 513 patients from the lead-in phase of the HALT-C Trial, PIIINP, HA, TIMP-1, and YKL-40 all correlated significantly to fibrosis stages. In a univariate analysis, the four variables were independent predictors of cirrhosis with HA having the highest odds ratio (Fontana et al. 2008). Later it was found that baseline levels of YKL-40, PIIINP, HA, and TIMP-1 assessed in the HALT-C Trial were associated with the risk of clinical outcome during follow-up. Interestingly, all markers except for PIIINP retained their significance when combined with other clinical parameters in a multivariate analysis (Fontana et al. 2010).

Other studies have also evaluated the prognostic values of these markers in patients with advanced liver disease. Baseline levels of PIIINP and YKL-40 have shown to be associated with clinical outcomes in patients with alcoholic liver disease, in which high levels of either of the two markers predicted shorter survival (Nojgaard et al. 2003). YKL-40 has also shown to correlate to stellate cell activation markers as well as rapid fibrosis progression in patients with recurrent HCV infection (Pungpapong et al. 2008). In 91 patients with HCV cirrhosis, HA had the highest predictive value for occurrence of severe complications and was equivalent to Child-Pugh score compared to other laboratory tests (Guechot et al. 2000).

A considerable limitation in validating novel biomarkers as prognostic markers is the lack of longitudinal studies with either double biopsies or clinical follow-up. Therefore current serum markers are not able to assess the dynamic changes in ECM remodeling which is required in accurate and early evaluation of novel anti-fibrotic drug efficacy (Patel and Shackel 2014).

Prognostic Protein Fingerprint Markers of Liver Fibrosis

The protein fingerprint markers have proven useful as diagnostic, prognostic, and efficacy of intervention markers in various liver diseases (Leeming et al. 2013b, 2014, 2015; Nielsen et al. 2015b, c; Schierwagen et al. 2013). In particularly one study by Nielsen et al. (2015c), our group demonstrated that two subtypes of the same protein provide different clinical information. The patients were from a prior multicenter, placebo-controlled phase II trial, in which its aim was to test the anti-fibrotic effect of a PPAR-γ activator, Farglitazar, in nonresponders of standard of care. Patients were given two different doses of Farglitazar or placebo twice a week for 52 weeks. Fibrosis was evaluated according to the Ishak score by liver biopsy at baseline and at week 52. The original trial found no effect of the drug; thus the trial was terminated (McHutchison et al. 2010).

In the study by Nielsen et al., we assessed two markers of formation and degradation of type III collagen (Pro-C3 and C3M) in baseline serum and stratified according to the baseline Ishak scores and found that the two markers had similar diagnostic performances as AST, ALT, and FibroTest. Interestingly, when stratifying the baseline marker levels to the changes in Ishak scores after 52 weeks, Pro-C3 was the only marker which significantly differentiated progressors from stable patients. Furthermore, based on the Pro-C3 baseline levels, patients with high levels of Pro-C3 had more than four times higher odds of being progressors of fibrosis than those with low baseline levels (Nielsen et al. 2015c).

This suggests that measurement of different end products of tissue destruction and measurement of either ECM remodeling or liver enzymes provide distinct information regarding the clinical utility of liver fibrosis biomarkers.

Applications to Other Diseases

Extracellular matrix remodeling is the common denominator in many connective tissue diseases; thus the ECM markers of liver diseases may be applied in other disorders as well. Most fibroproliferative disorders share a number of characteristics of abnormal ECM remodeling, including high matrix degradation, assembly, and disorganization. The high remodeling of ECM proteins is found in diseases such as:

- Rheumatoid arthritis affecting the cartilage and synovium (type I, II, and III collagens)
- Osteoarthritis affecting the articular cartilage (type II collagen and aggrecan)

- Metabolic bone diseases (type I collagen)
- Sarcopenia (type VI collagen)
- Cancer (basement membrane and desmoplasia)
- Atherosclerosis (type I and III collagens, titin, and versican)
- Other fibrotic diseases including the lung (elastin, type I, III, and V collagens) and kidney (basement membrane) (Karsdal et al. 2013)

The protein fingerprint markers provide a good example of how the same markers of ECM remodeling can be applied in two different fibrotic diseases. A panel of ECM formation and degradation markers was assessed in a study of 96 cirrhotic patients with portal hypertension. Most of the markers correlated to the degree of portal hypertension in these patients, and when combining several markers in an algorithm, the correlation to portal hypertension was improved (Leeming et al. 2013b, 2015). The same panel was assessed in another study of 217 patients with idiopathic pulmonary fibrosis. The serum levels of the markers were increased in patients compared to healthy individuals, but interestingly increased serum concentrations were associated with disease progression and the rate of this increase predicted survival (Jenkins et al. 2015).

Summary Points

- The hallmark of fibrosis is the accelerated accumulation of extracellular matrix proteins, ultimately leading to loss of organ function.
- There is a clinical need of earlier disease identification, of better treatment and prevention of complications to cirrhosis, and to identify fast progressors.
- Despite the limitations of liver biopsy, histological evaluation of the liver tissue remains the standard against which novel noninvasive methods are benchmarked.
- Direct markers are believed to reflect both the activity of the fibrotic processes and the total extracellular matrix undergoing remodeling.
- The most common extracellular matrix remodeling markers to stage the degree of liver fibrosis include hyaluronic acid, N-terminal procollagen type III, type IV collagen, and YKL-40.
- General for the direct extracellular matrix markers is the lack of assay standardization as well as variation in histological definitions and patient populations; thus it is difficult to draw strong conclusions about which biomarker performs best.
- The neo-epitope approach has received increased attention due to the diagnostic and prognostic potentials as serological biomarkers of extracellular matrix remodeling in bone and cartilage diseases and has in recent years been implemented in fibrotic diseases.
- Measurement of different end products of extracellular matrix remodeling may provide distinct information regarding the clinical utility of liver fibrosis biomarkers.

- At present, none of the available extracellular matrix remodeling biomarkers are yet able to replace biopsy in staging liver fibrosis or provide reliable assessment of fibrosis progression in chronic liver diseases.

References

Afdhal NH, Nunes D. Evaluation of liver fibrosis: a concise review. Am J Gastroenterol. 2004;99:1160–74.

Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: structure and function. Acta Biomater. 2009;5:1–13.

bdel-Aziz G, Rescan PY, Clement B, Lebeau G, Rissel M, Grimaud JA, Campion JP, Guillouzo A. Cellular sources of matrix proteins in experimentally induced cholestatic rat liver. J Pathol. 1991;164:167–74.

Bedossa P, Paradis V. Liver extracellular matrix in health and disease. J Pathol. 2003;200:504–15.

Boeker KH, Haberkorn CI, Michels D, Flemming P, Manns MP, Lichtinghagen R. Diagnostic potential of circulating TIMP-1 and MMP-2 as markers of liver fibrosis in patients with chronic hepatitis C. Clin Chim Acta. 2002;316:71–81.

Bruckner P. Suprastructures of extracellular matrices: paradigms of functions controlled by aggregates rather than molecules. Cell Tissue Res. 2010;339:7–18.

Cassiman D, Roskams T. Beauty is in the eye of the beholder: emerging concepts and pitfalls in hepatic stellate cell research. J Hepatol. 2002;37:527–35.

Chung L, Dinakarpandian D, Yoshida N, Lauer-Fields JL, Fields GB, Visse R, Nagase H. Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis. EMBO J. 2004;23:3020–30.

Dam EB, Byrjalsen I, Karsdal MA, Qvist P, Christiansen C. Increased urinary excretion of C-telopeptides of type II collagen (CTX-II) predicts cartilage loss over 21 months by MRI. Osteoarthritis Cartilage. 2009;17:384–9.

Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2002;2:161–74.

El-Mezayen HA, Habib S, Marzok HF, Saad MH. Diagnostic performance of collagen IV and laminin for the prediction of fibrosis and cirrhosis in chronic hepatitis C patients: a multicenter study. Eur J Gastroenterol Hepatol. 2015;27:378–85.

Exposito JY, Valcourt U, Cluzel C, Lethias C. The fibrillar collagen family. Int J Mol Sci. 2010;11:407–26.

Fanjul-Fernandez M, Folgueras AR, Cabrera S, Lopez-Otin C. Matrix metalloproteinases: evolution, gene regulation and functional analysis in mouse models. Biochim Biophys Acta. 2010;1803:3–19.

Fontana RJ, Goodman ZD, Dienstag JL, Bonkovsky HL, Naishadham D, Sterling RK, Su GL, Ghosh M, Wright EC. Relationship of serum fibrosis markers with liver fibrosis stage and collagen content in patients with advanced chronic hepatitis C. Hepatology. 2008;47:789–98.

Fontana RJ, Dienstag JL, Bonkovsky HL, Sterling RK, Naishadham D, Goodman ZD, Lok AS, Wright EC, Su GL. Serum fibrosis markers are associated with liver disease progression in non-responder patients with chronic hepatitis C. Gut. 2010;59:1401–9.

Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. J Cell Sci. 2010;123:4195–200.

Friedman SL. Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. N Engl J Med. 1993;328:1828–35.

Grenard P, Bresson-Hadni S, El AS, Chevallier M, Vuitton DA, Ricard-Blum S. Transglutaminase-mediated cross-linking is involved in the stabilization of extracellular matrix in human liver fibrosis. J Hepatol. 2001;35:367–75.

Gressner AM. Perisinusoidal lipocytes and fibrogenesis. Gut. 1994;35:1331–3.
Gressner OA, Gao C. Monitoring fibrogenic progression in the liver. Clin Chim Acta. 2014;433:111–22.
Gressner OA, Beer N, Jodlowski A, Gressner AM. Impact of quality control accepted inter-laboratory variations on calculated Fibrotest/Actitest scores for the non-invasive biochemical assessment of liver fibrosis. Clin Chim Acta. 2009;409:90–5.
Guechot J, Laudat A, Loria A, Serfaty L, Poupon R, Giboudeau J. Diagnostic accuracy of hyaluronan and type III procollagen amino-terminal peptide serum assays as markers of liver fibrosis in chronic viral hepatitis C evaluated by ROC curve analysis. Clin Chem. 1996;42:558–63.
Guechot J, Serfaty L, Bonnand AM, Chazouilleres O, Poupon RE, Poupon R. Prognostic value of serum hyaluronan in patients with compensated HCV cirrhosis. J Hepatol. 2000;32:447–52.
Heinegard D. Proteoglycans and more – from molecules to biology. Int J Exp Pathol. 2009;90:575–86.
Hu J, Van den Steen PE, Sang QX, Opdenakker G. Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. Nat Rev Drug Discov. 2007;6:480–98.
Idobe Y, Murawaki Y, Ikuta Y, Koda M, Kawasaki H. Post-prandial serum hyaluronan concentration in patients with chronic liver disease. Intern Med. 1998;37:568–75.
Jansen C, Leeming DJ, Mandorfer M, Byrjalsen I, Schierwagen R, Schwabl P, Karsdal MA, Anadol E, Strassburg CP, Rockstroh J, Peck-Radosavljevic M, Moller S, Bendtsen F, Krag A, Reiberger T, Trebicka J. PRO-C3-levels in patients with HIV/HCV-Co-infection reflect fibrosis stage and degree of portal hypertension. PLoS One. 2014;9:e108544.
Jenkins RG, Simpson JK, Saini G, Bentley JH, Russell AM, Braybrooke R, Molyneaux PL, McKeever TM, Wells AU, Flynn A, Hubbard RB, Leeming DJ, Marshall RP, Karsdal MA, Lukey PT, Maher TM. Longitudinal change in collagen degradation biomarkers in idiopathic pulmonary fibrosis: an analysis from the prospective, multicentre PROFILE study. Lancet Respir Med. 2015;3:462–72.
Johansen JS, Moller S, Price PA, Bendtsen F, Junge J, Garbarsch C, Henriksen JH. Plasma YKL-40: a new potential marker of fibrosis in patients with alcoholic cirrhosis? Scand J Gastroenterol. 1997;32:582–90.
Johansen JS, Christoffersen P, Moller S, Price PA, Henriksen JH, Garbarsch C, Bendtsen F. Serum YKL-40 is increased in patients with hepatic fibrosis. J Hepatol. 2000;32:911–20.
Karsdal MA, Henriksen K, Leeming DJ, Woodworth T, Vassiliadis E, Bay-Jensen AC. Novel combinations of Post-Translational Modification (PTM) neo-epitopes provide tissue-specific biochemical markers – are they the cause or the consequence of the disease? Clin Biochem. 2010;43:793–804.
Karsdal MA, Delvin E, Christiansen C. Protein fingerprints – relying on and understanding the information of serological protein measurements. Clin Biochem. 2011;44:1278–9.
Karsdal MA, Nielsen MJ, Sand JM, Henriksen K, Genovese F, Bay-Jensen AC, Smith V, Adamkewicz JI, Christiansen C, Leeming DJ. Extracellular matrix remodeling: the common denominator in connective tissue diseases. Possibilities for evaluation and current understanding of the matrix as more than a passive architecture, but a key player in tissue failure. ASSAY Drug Dev Technol. 2013;11:70–92.
Karsdal MA, Krarup H, Sand JM, Christensen PB, Gerstoft J, Leeming DJ, Weis N, Schaffalitzky de Muckadell OB, Krag A. Review article: the efficacy of biomarkers in chronic fibroproliferative diseases – early diagnosis and prognosis, with liver fibrosis as an exemplar. Aliment Pharmacol Ther. 2014;40:233–49.
Karsdal MA, Manon-Jensen T, Genovese F, Kristensen JH, Nielsen MJ, Sand JM, Hansen NU, Bay-Jensen AC, Bager CL, Krag A, Blanchard A, Krarup H, Leeming DJ, Schuppan D. Novel insights into the function and dynamics of extracellular matrix in liver fibrosis. Am J Physiol Gastrointest Liver Physiol. 2015;308:G807–30.
Kasahara A, Hayashi N, Mochizuki K, Oshita M, Katayama K, Kato M, Masuzawa M, Yoshihara H, Naito M, Miyamoto T, Inoue A, Asai A, Hijioka T, Fusamoto H, Kamada T. Circulating matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 as serum

markers of fibrosis in patients with chronic hepatitis C. Relationship to interferon response. J Hepatol. 1997;26:574–83.

Knittel T, Kobold D, Saile B, Grundmann A, Neubauer K, Piscaglia F, Ramadori G. Rat liver myofibroblasts and hepatic stellate cells: different cell populations of the fibroblast lineage with fibrogenic potential. Gastroenterology. 1999;117:1205–21.

Koivisto H, Hietala J, Niemela O. An inverse relationship between markers of fibrogenesis and collagen degradation in patients with or without alcoholic liver disease. Am J Gastroenterol. 2007;102:773–9.

Lee WM, Dienstag JL, Lindsay KL, Lok AS, Bonkovsky HL, Shiffman ML, Everson GT, Di Bisceglie AM, Morgan TR, Ghany MG, Morishima C, Wright EC, Everhart JE. Evolution of the HALT-C Trial: pegylated interferon as maintenance therapy for chronic hepatitis C in previous interferon nonresponders. Control Clin Trials. 2004;25:472–92.

Leeming DJ, Byrjalsen I, Jimenez W, Christiansen C, Karsdal MA. Protein fingerprinting of the extracellular matrix remodelling in a rat model of liver fibrosis – a serological evaluation. Liver Int. 2013a;33:439–47.

Leeming DJ, Karsdal MA, Byrjalsen I, Bendtsen F, Trebicka J, Nielsen MJ, Christiansen C, Moller S, Krag A. Novel serological neo-epitope markers of extracellular matrix proteins for the detection of portal hypertension. Aliment Pharmacol Ther. 2013b;38:1086–96.

Leeming DJ, Anadol E, Schierwagen R, Karsdal MA, Byrjalsen I, Nielsen MJ, Schwarzer-Zander C, Boesecke C, Bendtsen F, Oller SM, Strassburg CP, Spengler U, Krag A, Rockstroh J, Trebicka J. Combined antiretroviral therapy attenuates hepatic extracellular matrix remodeling in HIV patients assessed by novel protein fingerprint markers. AIDS. 2014;28:2081–90.

Leeming DJ, Veidal SS, Karsdal MA, Nielsen MJ, Trebicka J, Busk T, Bendtsen F, Krag A, Moller S. Pro-C5, a marker of true type V collagen formation and fibrillation, correlates with portal hypertension in patients with alcoholic cirrhosis. Scand J Gastroenterol. 2015;50:584–92.

Leroy V, De TC, Barnoud R, Hartmann JD, Baud M, Ouzan D, Zarski JP. Changes in histological lesions and serum fibrogenesis markers in chronic hepatitis C patients non-responders to interferon alpha. J Hepatol. 2001;35:120–6.

Manning DS, Afdhal NH. Diagnosis and quantitation of fibrosis. Gastroenterology. 2008;134: 1670–81.

Marinho CC, Bretas T, Voieta I, Queiroz LC, Ruiz-Guevara R, Teixeira AL, Antunes CM, Prata A, Lambertucci JR. Serum hyaluronan and collagen IV as non-invasive markers of liver fibrosis in patients from an endemic area for schistosomiasis mansoni: a field-based study in Brazil. Mem Inst Oswaldo Cruz. 2010;105:471–8.

McHutchison J, Goodman Z, Patel K, Makhlouf H, Rodriguez-Torres M, Shiffman M, Rockey D, Husa P, Chuang WL, Levine R, Jonas M, Theodore D, Brigandi R, Webster A, Schultz M, Watson H, Stancil B, Gardner S. Farglitazar lacks antifibrotic activity in patients with chronic hepatitis C infection. Gastroenterology. 2010;138:1365–73. 1373.

Mehta SH, Lau B, Afdhal NH, Thomas DL. Exceeding the limits of liver histology markers. J Hepatol. 2009;50:36–41.

Murawaki Y, Ikuta Y, Nishimura Y, Koda M, Kawasaki H. Serum markers for connective tissue turnover in patients with chronic hepatitis B and chronic hepatitis C: a comparative analysis. J Hepatol. 1995;23:145–52.

Murawaki Y, Ikuta Y, Koda M, Yamada S, Kawasaki H. Comparison of serum 7S fragment of type IV collagen and serum central triple-helix of type IV collagen for assessment of liver fibrosis in patients with chronic viral liver disease. J Hepatol. 1996;24:148–54.

Murawaki Y, Ikuta Y, Okamoto K, Koda M, Kawasaki H. Diagnostic value of serum markers of connective tissue turnover for predicting histological staging and grading in patients with chronic hepatitis C. J Gastroenterol. 2001;36:399–406.

Nielsen MJ, Kazankov K, Leeming DJ, Karsdal MA, Barrera F, McLeod D, George J, Gronbaek H. Markers of collagen remodeling detect clinically significant fibrosis in chronic hepatitis C patients. PLoS One. 2015a;10:e0137302.

Nielsen MJ, Lehmann J, Leeming DJ, Schierwagen R, Klein S, Jansen C, Strassburg CP, Bendtsen F, Moller S, Sauerbruch T, Karsdal MA, Krag A, Trebicka J. Circulating elastin fragments are not affected by hepatic, renal and hemodynamic changes, but reflect survival in cirrhosis with TIPS. Dig Dis Sci. 2015b;60:3456–64.

Nielsen MJ, Veidal SS, Karsdal MA, Orsnes-Leeming DJ, Vainer B, Gardner SD, Hamatake R, Goodman ZD, Schuppan D, Patel K. Plasma Pro-C3 (N-terminal type III collagen propeptide) predicts fibrosis progression in patients with chronic hepatitis C. Liver Int. 2015c;35:429–37.

Nojgaard C, Johansen JS, Christensen E, Skovgaard LT, Price PA, Becker U. Serum levels of YKL-40 and PIIINP as prognostic markers in patients with alcoholic liver disease. J Hepatol. 2003;39:179–86.

Oberti F, Valsesia E, Pilette C, Rousselet MC, Bedossa P, Aube C, Gallois Y, Rifflet H, Maiga MY, Penneau-Fontbonne D, Cales P. Noninvasive diagnosis of hepatic fibrosis or cirrhosis. Gastroenterology. 1997;113:1609–16.

Overall CM. Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites. Mol Biotechnol. 2002;22:51–86.

Parkes J, Roderick P, Harris S, Day C, Mutimer D, Collier J, Lombard M, Alexander G, Ramage J, Dusheiko G, Wheatley M, Gough C, Burt A, Rosenberg W. Enhanced liver fibrosis test can predict clinical outcomes in patients with chronic liver disease. Gut. 2010;59:1245–51.

Patel K, Shackel NA. Current status of fibrosis markers. Curr Opin Gastroenterol. 2014;30:253–9.

Pinzani M. The ELF, panel: a new crystal ball in hepatology? Gut. 2010;59:1165–7.

Pungpapong S, Nunes DP, Krishna M, Nakhleh R, Chambers K, Ghabril M, Dickson RC, Hughes CB, Steers J, Nguyen JH, Keaveny AP. Serum fibrosis markers can predict rapid fibrosis progression after liver transplantation for hepatitis C. Liver Transpl. 2008;14:1294–302.

Radwan MI, Pasha HF, Mohamed RH, Hussien HI, El-Khshab MN. Influence of transforming growth factor-beta1 and tumor necrosis factor-alpha genes polymorphisms on the development of cirrhosis and hepatocellular carcinoma in chronic hepatitis C patients. Cytokine. 2012;60:271–6.

Ramadori G, Saile B. Portal tract fibrogenesis in the liver. Lab Investig. 2004;84:153–9.

Ramadori G, Zohrens G, Manns M, Rieder H, Dienes HP, Hess G, Meyer KH, Buschenfelde Z. Serum hyaluronate and type III procollagen aminoterminal propeptide concentration in chronic liver disease. Relationship to cirrhosis and disease activity. Eur J Clin Invest. 1991;21:323–30.

Reijman M, Hazes JM, Bierma-Zeinstra SM, Koes BW, Christgau S, Christiansen C, Uitterlinden AG, Pols HA. A new marker for osteoarthritis: cross-sectional and longitudinal approach. Arthritis Rheum. 2004;50:2471–8.

Ricard-Blum S, Ruggiero F. The collagen superfamily: from the extracellular matrix to the cell membrane. Pathol Biol (Paris). 2005;53:430–42.

Rosenberg WM, Voelker M, Thiel R, Becka M, Burt A, Schuppan D, Hubscher S, Roskams T, Pinzani M, Arthur MJ. Serum markers detect the presence of liver fibrosis: a cohort study. Gastroenterology. 2004;127:1704–13.

Schaller S, Henriksen K, Hoegh-Andersen P, Sondergaard BC, Sumer EU, Tanko LB, Qvist P, Karsdal MA. In vitro, ex vivo, and in vivo methodological approaches for studying therapeutic targets of osteoporosis and degenerative joint diseases: how biomarkers can assist? ASSAY Drug Dev Technol. 2005;3:553–80.

Schierwagen R, Leeming DJ, Klein S, Granzow M, Nielsen MJ, Sauerbruch T, Krag A, Karsdal MA, Trebicka J. Serum markers of the extracellular matrix remodeling reflect antifibrotic therapy in bile-duct ligated rats. Front Physiol. 2013;4:195.

Schuppan D. Structure of the extracellular matrix in normal and fibrotic liver: collagens and glycoproteins. Semin Liver Dis. 1990;10:1–10.

Schuppan D, Kim YO. Evolving therapies for liver fibrosis. J Clin Invest. 2013;123:1887–901.

Schuppan D, Ruehl M, Somasundaram R, Hahn EG. Matrix as a modulator of hepatic fibrogenesis. Semin Liver Dis. 2001;21:351–72.

Tran A, Benzaken S, Saint-Paul MC, Guzman-Granier E, Hastier P, Pradier C, Barjoan EM, Demuth N, Longo F, Rampal P. Chondrex (YKL-40), a potential new serum fibrosis marker in patients with alcoholic liver disease. Eur J Gastroenterol Hepatol. 2000;12:989–93.

Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res. 2003;92:827–39.

Walsh KM, Timms P, Campbell S, MacSween RN, Morris AJ. Plasma levels of matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases -1 and -2 (TIMP-1 and TIMP-2) as noninvasive markers of liver disease in chronic hepatitis C: comparison using ROC analysis. Dig Dis Sci. 1999;44:624–30.

Xie SB, Yao JL, Zheng RQ, Peng XM, Gao ZL. Serum hyaluronic acid, procollagen type III and IV in histological diagnosis of liver fibrosis. Hepatobiliary Pancreat Dis Int. 2003;2:69–72.

Yamada M, Fukuda Y, Koyama Y, Nakano I, Urano F, Katano Y, Hayakawa T. Serum hyaluronic acid reflects the effect of interferon treatment on hepatic fibrosis in patients with chronic hepatitis C. J Gastroenterol Hepatol. 1996;11:646–51.

Yurchenco PD, Schittny JC. Molecular architecture of basement membranes. FASEB J. 1990;4:1577–90.

Zhu CL, Li WT, Li Y, Gao RT. Serum levels of tissue inhibitor of metalloproteinase-1 are correlated with liver fibrosis in patients with chronic hepatitis B. J Dig Dis. 2012;13:558–63.

Interaction of Sialyltransferases, Sialidases, and Sialic Acids in Liver Diseases and Applications to Biomarker Discovery

11

A. Ata Alturfan and Ebru Emekli-Alturfan

Contents

Abstract

Sialic acids are family of extraordinary monosaccharides with nine-carbon backbone that are thoroughly expressed on all cell surfaces of vertebrates and higher

A.A. Alturfan (✉)
Department of Sciences, Institute of Forensic Sciences, Istanbul University, Istanbul, Turkey
e-mail: ataalturfan@gmail.com

E. Emekli-Alturfan (✉)
Department of Biochemistry, Faculty of Dentistry, Marmara University, Istanbul, Turkey
e-mail: ebruemekli@yahoo.com

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_19

invertebrates and also on specific bacteria that interact with vertebrates. In liver diseases, alterations in the carbohydrate content of plasma glycoproteins have been described either due to tissue destruction, tissue proliferation, depolymerization, or inflammation. Accordingly, sialic acid is being studied in liver diseases since the last two decades. Sialyltransferase group of enzymes catalyzes the transfer of the activated form of sialic acid onto the acceptor sugar. Sialidases (EC 3.2.1.18, also called neuraminidases) are glycosidases that catalyze the removal of sialic acid residues linked α-glycosidically from carbohydrate groups of glycoproteins and glycolipids. The sialyltransferases, sialidases, and sialic acids have been evaluated in liver diseases, and significant alterations have been found. Detailed understanding of the role of sialic acid, sialidases, and sialyltransferases and their interactions in liver diseases may significantly contribute to open new exciting frontiers of basic and therapeutic exploration.

Keywords
Sialic acid • Sialyltransferases • Sialidases • Liver disease • Biomarkers

List of Abbreviations

Apo	Apolipoprotein
CMAH	Cytidine monophosphate acetylneuraminic acid hydroxylase
CMP	Cytidine monophosphate
CRP	C reactive protein
FSA	Free sialic acid
HCC	Hepatocellular carcinoma
LSA	Lipid-bound sialic acid
NEU1	Neurominidase 1
NEU2	Neurominidase 2
NEU3	Neurominidase 3
NEU4	Neurominidase 4
Neu5Ac	*N*-acetylneuraminic acid
OJM	Obstructive jaundice model
PEG	Polyethylene glycol
PSA	Polysialic acids
SOC	Sham operated control
ST6Gal I	Galactoside α2,6 sialyltranferase 1
TSA	Total sialic acid
vWF	Von Willebrand factor

Key Facts of Sialyltransferases in Liver Disease

1. Sialyltransferases catalyze the transfer of the activated form of sialic acid onto the acceptor sugar and catalyze the formation of different linkages and differ in their acceptor specificities.

2. Sialyltransferases have the similar architecture, and vertebrate sialyltransferases are membrane-bound proteins with the catalytic domain facing into the lumen.
3. More than 20 different sialyltransferases are involved in the biosynthesis of sialylated glycoproteins and glycolipids, and they are encoded by the human genome.
4. Sialyltransferase enzymes differ in their substrate specificity, tissue distribution, and various biochemical parameters.
5. In early years, it has been reported that serum from a patient with α-1-antitrypsin deficiency and hepatic cirrhosis was substantially deficient in sialyltransferase.
6. The serum sialyltransferase deficiency in patients arose after chronic and extensive liver disease involving hepatic accumulation of α-1-antitrypsin rather than the enzyme deficiency as the major cause of the hepatic cirrhosis and α-1-antitrypsin deficiency.
7. Plasma sialyltransferase has been suggested to be a useful marker enzyme for monitoring effectiveness of therapeutic programs for disseminated neoplasms.
8. Altered sialylation has been associated with metastatic cell behaviors like invasion and enhanced cell survival and the sialic acid linkage to other sugars, existing in three main configurations, α2,3, α2,6, and α2,8, catalyzed by sialyltransferases.

Key Facts of Sialidases in Liver Disease

1. There are four types of mammalian sialidases that have been identified and characterized as NEU1, NEU2, NEU3, and NEU4.
2. In humans, the identity of NEU1 to other sialidases is low, but NEU2, NEU3, and NEU4 show higher homology to each other, and their substrate specificities are also different.
3. Sialidases have been suggested to be candidate therapeutic agents for some diseases such as spinal cord injury and for future approaches and therapeutic purposes.
4. It has been reported that a major fraction of sialidase with activity toward ganglioside substrate was localized in the plasma membrane of the liver cell.
5. Decreased NEU1 expression has been shown in various cancers, and there is an inverse relationship between NEU1 expression level and metastatic ability.
6. Liver NEU3 overexpression has been shown to positively improve insulin sensitivity and glucose tolerance in C57BL/6 and insulin-resistant mice by increased deposition of glycogen and triglycerides.
7. Neuraminidase inhibitors are another important subject related to sialidases with some adverse effects of their use have been reported.

Definitions of Words and Terms

Acute phase response	The acute phase response is a nonspecific response to tissue injury or infection which affects several organs and tissues.
Bioavailability	It is the proportion of the administered substance available for use or storage and capable of being absorbed.
Cirrhosis	Cirrhosis is a late stage of scarring or fibrosis of the liver caused by many forms of liver diseases and conditions like hepatitis and chronic alcohol abuse.
CRP	C-reactive protein is a major component of the acute phase response. It is synthesized in the liver, and it is believed to mediate binding of foreign polysaccharides and phospholipids and also activate complement via the classic pathway.
Fibrinogen	It is a globulin of the blood plasma, converted into fibrin by the action of thrombin in the presence of calcium to produce coagulation.
Glomerular filtration	Glomerular filtration is the process by which the kidneys filter the blood in order to remove excess wastes and fluids.
Glycolipid	A lipid containing carbohydrate groups.
Glycoprotein	A class of conjugated proteins consisting of a compound of protein with a carbohydrate group.
Half-life	Half-life is the amount of time required for the amount of something to fall to half its initial value.
Inflammation	A localized protective response emerged by injury or destruction of tissues, serving to destroy both the injurious agent and the injured tissue.
Orosomucoid	Orosomucoid (ORM) or alpha-1-acid glycoprotein is an acute phase and is synthesized primarily in hepatocytes.
Pegylation	Pegylation is the term used to improve the pharmacokinetic properties of many biopharmaceutical proteins aiming to increase the elimination half-life and stability of the protein in question.
Sialic	Pertaining to the saliva.
Sialidases	They are glycosidases that catalyze the removal of sialic acid residues linked α-glycosidically from carbohydrate groups of glycoproteins and glycolipids.
Sialylation	Sialylation is the introduction of additional sialic acid residues to a protein.
Sialyltransferase	They are group of enzymes that catalyze the transfer of the activated form of sialic acid onto the acceptor sugar.

Introduction

Sialic acids are a different family of extraordinary monosaccharides that are thoroughly expressed on all cell surfaces of vertebrates and so-called "higher" invertebrates and also on specific bacteria that interact with vertebrates (Varki and Gagneux 2012). There are over 50 natural analogues of sialic acid resulting from modifications occurring at the sialic acid backbone, such as the introduction of lactoyl, sulfate, methyl, and phosphate groups at the hydroxyl groups at C-4, C-7, C-8, and C-9. The most common sialic acid derivatives, *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), 2-keto-3-deoxy-D-glycero-D-galacturononic acid, and neuraminic acid (Neu), are formed by the solitary substitution at C-5. Polysialic acids (PSA) are produced by the interaction of the residues of Neu5Ac with each other in a conjugation process (Fig. 1). These homopolymers may be referred to as colominic acids and are added posttranslationally to a glycoprotein on the surface of glial cells and the skeletal muscle called neuronal

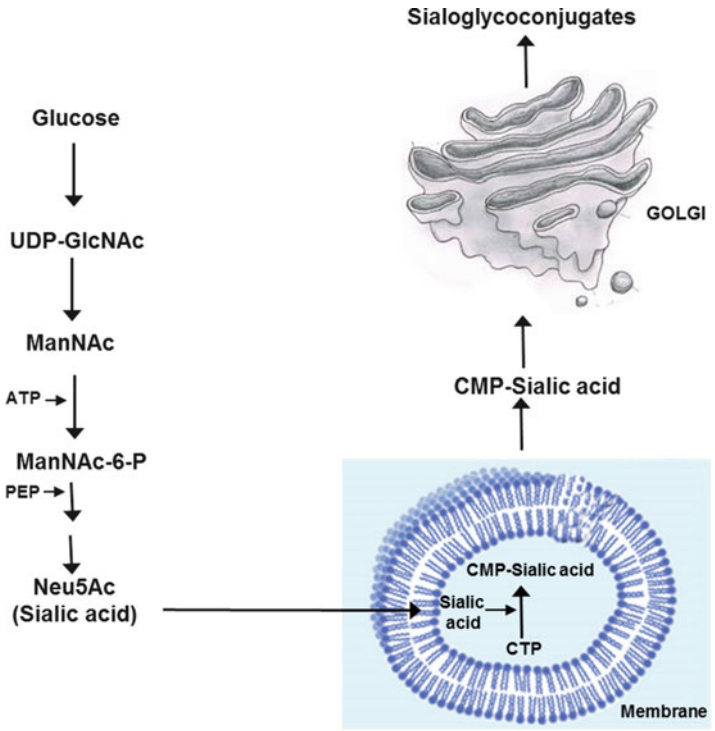

Fig. 1 Graphic illustrates the biosynthetic pathway of sialic acid. Synthesis of sialic acid from glucose and formation of sialoglycoconjugates. *CTP* cytidine triphosphate, *CMP* cytidine monophosphate

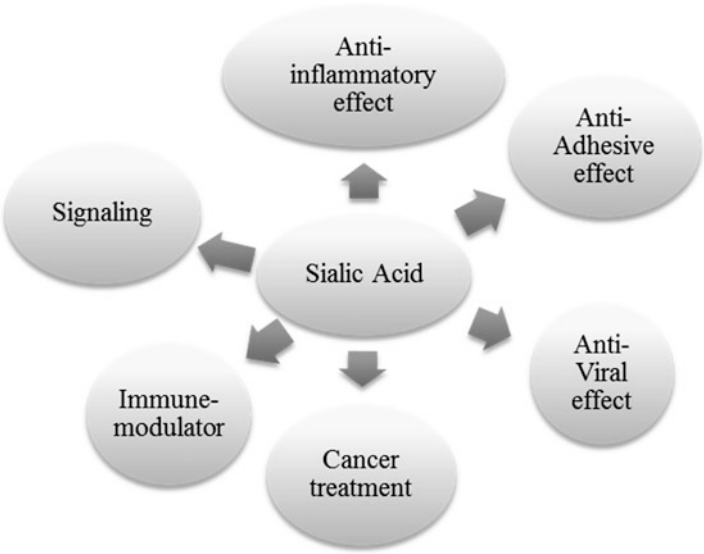

Fig. 2 Major functions of sialic acid. Major functions and effects of sialic acid are listed

cell adhesion molecule. Sialic acids may participate in several neurological processes, including the maintenance of plasticity during neuron development, fasciculation, and axonal branching by regulating homophilic interactions through this association. The attraction and repulsion of charged molecules and other entities are maintained by the negative charge at C-1 (Traving and Schauer 1998; Mühlenhoff 1998; Gruszewska and Chrostek 2016). Major functions of sialic acids are given in Fig. 2 (Varki 2008), and the main enzyme groups and their functions in sialic acid metabolism are given in Tables 1, 2, and 3 (Traving and Schauer 1998).

The term sialylation is the introduction of additional sialic acid residues to a protein. Sialylation has been defined as an effective alternative to pegylation and being not as challenging technically as pegylation. Pegylation is used to improve the pharmacokinetic properties of many biopharmaceutical proteins aiming to increase the elimination half-life and stability of the protein in question. Increasing the overall size of the circulating protein is one modification as molecules that are not associated with plasma-based proteins and with molecular weights <5 kDa tend to be excreted rapidly via the kidneys (Werle and Bernkop-Schnürch 2006). As the rate of glomerular filtration is reduced due to this enlargement, the protein has more time to interact with the target tissue or antigen. Pegylation is used for such increases in size involving the covalent attachment of either linear or branched chains of polyethylene glycol via a chemically reactive side chain, including a hydroxyl succinimidyl ester or an aldehyde group, to link to either the alpha or the epsilon amino groups on the protein (Byrne et al. 2007). On the other hand, oversialylation can increase the malignancy and metastatic potential of tumor cells by protecting cells from humoral or cellular defense systems as often observed in tumor cells or on placental

Table 1 Group of enzymes in sialic acid metabolism. The main enzyme groups with their functions in sialic acid metabolism are given as sialyltransferase, cytidine monophosphate (CMP) acetylneuraminic acid hydroxylase (CMAH), sialate-*O*-methyltransferase, sialate-*O*-acetyltransferase, sialate-*O*-acetylesterase, acetylneuraminate lyase, and sialidase

Enzymes in sialic acid metabolism and their functions	
Sialyltransferase	Sialyltransferases catalyze the transfer of CMP-activated sialic acid molecules onto oligosaccharides, glycolipids, and glycoproteins
Cytidine monophosphate (CMP)-acetylneuraminic acid hydroxylase (CMAH)	CMAH converts CMPNeu5Ac into CMP-Neu5Gc
Sialate-*O*-methyltransferase	Sialate-*O*-methyltransferase enzyme is responsible for the sialate methylation by transferring a methyl group from S-adenosylmethionine onto position 8 of sialic acids, and it is also unique for glycoconjugate-bound sialic acids
Sialate-*O*-acetyltransferase	Sialate-*O*-acetyltransferase catalyzes *O*-acetylation of specific positions on sialic acids
Sialate-*O*-acetylesterase	Sialate-*O*-acetylesterase belongs to the family of hydrolase and catalyzes the removal of *O*-acetyl ester groups from position 9 of the parent sialic acid, *N*-acetylneuraminic acid
Acetylneuraminate lyase	Acetylneuraminate lyase is a cytosolic enzyme that splits acetylneuraminic acids into acetylmannosamines and pyruvate
Sialidase	Sialidase detaches the sialic acid residues from the cell surface or sialoglycoconjugates

Table 2 General properties of sialyltransferase group of enzymes. Localization, structure, and types and function of sialyltransferase group of enzymes (Harduin-Lepers et al. 2001, 2005)

Sialyltransferase group of enzymes	
Localization	They predominantly reside in the *trans*-golgi compartment
Structure	They have a short N-terminal cytoplasmic tail, a transmembrane domain, and a stem region of variable length from 20 to 200 amino acids followed by a large C-terminal catalytic domain
Types	Nearly 20 individual sialyltransferase enzymes have been proposed
Function	They catalyze the transfer of CMP-NeuNAc onto the acceptor sugar and the formation of different linkages (α2,3, α2,6, and α2,8)

syncytioblasts (Varki 2008). Microorganisms that coat themselves by colominic acid, a PSA, apply a similar antirecognition strategy that allows better survival in the host and thus enhances virulence. Group B *Streptococcus* is an example presenting terminal Sia α2,3-Gal b1,4 GlcNAc units, similar to human neutrophils that interact in the cell membrane with siglec-9 in *cis*. The binding of bacterial oligosaccharide in *trans* to the same neutrophil siglec-9 in a Sia-dependent manner has been shown to result in the weakening of the neutrophil immune response, thus demonstrating a new mechanism of bacterial immune evasion (Carlin et al. 2009).

Table 3 General properties of the mammalian sialidases. Some properties of the mammalian sialidases, their substrates, primary localization in cells, and optimum pH (Miyagi and Yamaguchi 2012)

	NEU 1	NEU 2	NEU 3	NEU 4
Substrates	Oligosaccharides	Oligosaccharides	Gangliosides	Oligosaccharides
	Glycopeptides	Glycopeptides		Glycopeptides
		Gangliosides		Gangliosides
Primary subcellular localization	Lysosomes	Cytosol	Plasma membranes	Lysosomes, mitochondria, and endoplasmic reticulum
Optimal pH	4.4–4.6	6.0–6.5	4.5–4.7	4.5–4.7

Sialic Acid in Liver Diseases

Changes in the carbohydrate content of plasma glycoproteins have been described in patients with different liver diseases. Sialic acid in the human serum has been shown to be higher in a number of pathological states where the pathology is either due to tissue destruction, tissue proliferation, depolymerization, or inflammation. Accordingly, sialic acid is being studied in liver diseases since the last two decades (O'Kennedy et al. 1991; Okude et al. 1993, 1995; Lindberg et al. 1997; Arif 2005; Alturfan et al. 2014). In 1978, Martinez et al. reported abnormal sialic acid content of the dysfibrinogenemia associated with liver diseases. They reported that sialic acid content of the purified fibrinogen was 12.7–71.4% higher in patients when compared to controls (Martinez et al. 1978). Variation in serum sialic acid level in a variety of inflammatory liver diseases is an important diagnostic and prognostic tool. In liver cirrhosis, liver cancer, viral hepatitis, fatty liver, and hepatoma, abnormal sialic acid levels have been reported. On the other hand, sialic acid levels were much higher than the upper range in metastatic liver cancer (Matsuzaki et al. 1981).

Arif et al. (2005) carried out a study to evaluate levels of sialic acid in the patients with liver cirrhosis, and they reported a marked increase of sialic acid level in the blood. They reported increased serum sialic acid in advanced and terminal stages of liver cirrhosis in patients who had developed complications of the disease. On the other hand, the level was normal in the early stage of disease in the patients who had no complications. In previous studies conducted in hepatobiliary diseases, it has been indicated that an increase in sialic acid level may occur in the patients suffering from viral hepatitis, liver cirrhosis, inflammation of biliary tract, and malignant neoplasms of the liver (Carlson 1980; Narvaiza et al. 1986; Gruszewska et al. 2014).

The elevation of sialic acid content has been suggested to be a consequence of liver damage resulting in abnormal carbohydrate composition of the fibrinogen since fibrinogen contains 0.6% sialic acid and fibrinogen and sialic acid are both acute phase reactants (Arif 2005). In another study in liver cirrhosis and viral hepatitis, a

decrease in sialoprotein has been reported that varied with the course of disease (Kaniak et al. 1980).

Sialic acid or *N*-acetylneuraminic is a component of the terminal part of some acute phase proteins like α-1-antichemotrypisin, α-1-antitrypsin, haptoglobin, and orosomucoid. Seventy percent of plasma sialic acid concentration might be explained by these glucoproteins. The production of sialic acid in hepatocytes is stimulated in inflammation and metabolic/oxidative stress situations. Therefore, sialic acid might be considered as a biomarker of serum concentration of many acute phase proteins and could be considered a systemic inflammatory biomarker since it could predict the risk for type 2 diabetes and cardiovascular diseases. Sialic acid and α-1-antitrypsin and the C-terminal fragment of α-1-antitrypsin, ceruloplasmin, fibrinogen, haptoglobin, homocystein, and plasminogen activator inhibitor-1 are among the hepatic biomarkers of inflammation related to the atherosclerotic process (Pinheiro et al. 2015).

Increased sialic acid levels have been suggested to reflect generalized endothelial cell dysfunction or macrovascular disease either through loss of sialic acid containing glycoproteins from vascular cells into the bloodstream or through an acute phase response. In liver destruction, sialic acid level rises proportional to hepatic damage since most of the circulating sialic acid is covalently attached to glycoproteins and more than 50% of total sialic acid comes from acute phase proteins such as α-acid glycoproteins, α-antitrypsin and fibrinogen, factor VII antigen, and activation markers of coagulation (Arif et al. 2005).

Authors aimed to investigate the sialic acid concentrations as total sialic acid (TSA), lipid-bound SA (LSA), and free SA (FSA) levels in the sera in liver cirrhosis in relation with the severity of liver disease. They found that the sialylation of serum proteins and lipids changes in liver cirrhosis, but only the serum concentrations of FSA are stage related and reflect the severity of liver disease (Chrostek et al. 2014; Gruszewska et al. 2014).

Other researchers carried out a study to investigate oxidant-antioxidant status and serum total sialic acid levels as alternative markers complementary to routine laboratory tests in an experimental obstructive jaundice model (OJM). Accordingly, rats were divided into three groups: sham-operated control (SOC), OJM monitored for 7 days (OJM-7), and OJM monitored for 14 days (OJM-14). We found that in both OJM groups, sialic acid and C-reactive protein (CRP) levels were significantly increased when compared with the SOC group. Moreover, sialic acid and CRP levels were significantly correlated in both groups. Therefore, we concluded that serum sialic acid may serve as an adjunct when combined with other markers in disease screening and progression as a marker of inflammation and oxidative stress in obstructive jaundice (Alturfan et al. 2014).

General Information on Sialyltransferases

Sialyltransferase group of enzymes catalyzes the transfer of the activated form of sialic acid, CMP-NeuNAc, onto the acceptor sugar. They catalyze the formation of different linkages (α2,3, α2,6, and α2,8) and differ in their acceptor specificities.

Having the similar architecture, all vertebrate sialyltransferases are type II transmembrane glycoproteins that predominantly reside in the *trans*-golgi compartment. They are membrane-bound proteins with the catalytic domain facing into the lumen. Sialyltransferases have a short N-terminal cytoplasmic tail, a unique transmembrane domain, and a stem region of variable length from 20 to 200 amino acids followed by a large C-terminal catalytic domain. A specific enzyme catalyzes each transfer reaction, and for that reason, nearly 20 individual sialyltransferase enzymes have been proposed. Although the reactions catalyzed are very similar, all of the sialyltransferase enzymes cloned to date exhibit very little homology except for a short consensus sequence that is called the sialyl motif, to which the activated sugar donor is suggested to bind (Breen et al. 1998; Harduin-Lepers et al. 2001, 2005).

More than 20 different sialyltransferases involved in the biosynthesis of sialylated glycoproteins and glycolipids have been reported to be encoded by the human genome; however, only 15 different human sialyltransferase cDNAs have been cloned and characterized. Sialyltransferase genes are differentially expressed in a tissue-, cell type-, and stage-specific manner in order to regulate the sialylation pattern of cells. Accordingly, sialyltransferase enzymes differ in their substrate specificity, tissue distribution, and various biochemical parameters. On the other hand, one linkage has been suggested to be synthesized by multiple enzymes as conducted by enzymatic analysis (Harduin-Lepers et al. 2001).

Sialyltransferases in Liver Disease

In early years, it has been reported that serum from a patient with α-1-antitrypsin deficiency and hepatic cirrhosis was substantially deficient in sialyltransferase (EC 2.4.99.1), which is described as an enzyme transferring sialic acid from cytidine 5′-monophosphate-*N*-acetylneuraminic acid to a variety of asialoglycoprotein acceptors. The same authors extended their studies and included serum from five additional patients with α-1-antitrypsin deficiency and juvenile hepatic cirrhosis as well as a liver specimen obtained at an autopsy of one of these patients. They reported the sialyltransferase activity in serum from six patients with α-1-antitrypsin deficiency and hepatic cirrhosis to be 50% of healthy pediatric control values and 30% of pediatric patients with liver disease. On the other hand, interestingly the serum of family members homozygous for α-1-antitrypsin deficiency but without hepatic cirrhosis, and serum of patients with a variety of other kinds of liver disease, failed to exhibit the marked sialyltransferase deficiency. They carried out their study with similar assays on a liver sample homogenate obtained from a patient with α-1-antitrypsin deficiency and hepatic cirrhosis and found out that the deficiency of sialyltransferase activity was not demonstrable in the liver. The authors suggested that the serum sialyltransferase deficiency in such patients probably arose after chronic and extensive liver disease involving hepatic accumulation of α-1-antitrypsin rather than the enzyme deficiency as the major cause of the hepatic cirrhosis and α-1-antitrypsin deficiency (Kuhlenschmidt et al. 1976).

In another early study, Henderson and Kessel measured the levels of sialyltransferase activity in the plasma of patients with neoplastic disease and

found elevated above-normal control values in 85% of patients that were examined. Moreover, there was a correlation between enzyme levels and course of disease in 46 of 57 patients studied serially during therapy. The authors concluded that plasma sialyltransferase could be a useful marker enzyme for monitoring effectiveness of therapeutic programs for disseminated neoplasms (Henderson and Kessel 1977).

Long-term ethanol has been shown to downregulate Gal β-1, 4GlcNAcα2, and 6-sialyltransferase (ST6Gal1), leading to defective glycosylation of a number of proteins including apolipoprotein E and apo J and the appearance of asialoconjugates in the blood of continuously alcohol-fed animals also in human alcoholics. The same authors explored the possibility of whether ethanol-induced downregulation of ST6Gal1 could contribute toward alcoholic steatosis in human alcoholics because of impaired lipid and lipoprotein transport caused by this downregulation. They suggested that alcohol-mediated downregulation of hepatic ST6Gal1 gene led to defective glycosylation of lipid-carrying apolipoproteins such as apo E and apo J, resulting in defective intracellular lipid and lipoprotein transport, which in turn may contribute to alcoholic steatosis (Gong et al. 2008).

Cao et al. suggested that sialyltransferases by sialylating plasma glycoproteins in hepatocytes may constitute markers for liver diseases. In their study, they examined the expression of the prevalent α2,6 sialyltransferase and sialoglycans in normal liver, cirrhotic liver, and hepatocellular carcinoma (HCC). They reported in normal and cirrhotic liver ST6Gal1 and sialoglycans were localized in the golgi region of hepatocytes surrounding the bile canaliculi and along the bile canaliculi, respectively. They showed the expression patterns of ST6Gal1 and sialoglycans in various liver tissues and demonstrated an altered expression of these structures between benign and malignant hepatocellular lesions (Cao et al. 2002).

Changes in glycosylation are accepted as a common feature of cancer cells. Altered sialylation has been associated with metastatic cell behaviors like invasion and enhanced cell survival, and the sialic acid linkage to other sugars, existing in three main configurations, α2,3, α2,6, and α2,8, catalyzed by sialyltransferases, has also suggested to be altered. All three configurations have been shown to be aberrantly expressed in cancer progression, with the increased α2,6 sialylation catalyzed by β-galactoside α2,6-sialyltranferase 1 (ST6Gal1), being frequent in many types of the cancers (Lu and Gu 2015).

General Information on Sialidases

Sialidases (EC 3.2.1.18, also called neuraminidases) are glycosidases that catalyze the removal of sialic acid residues linked α-glycosidically from carbohydrate groups of glycoproteins and glycolipids. Based on their substrate specificity and catalytic mechanism, sialidases can be separated into three different classes. Hydrolytic sialidases cleave the glycosidic bond of terminal sialic acids and release free sialic acid. Trans-sialidases' role is to transfer the cleaved sialic acid to other glycoconjugates. Both classes belong to exo-α-sialidases (EC 3.2.1.18). Hydrolytic sialidases usually have a wide substrate specificity and cleave α2,3-, α2,6-, and α2,8-linked terminal sialic

acids; however, trans-sialidases prefer α2,3-linked substrates. **IT**-sialidase (EC 4.2.2.15) is the third class that is strictly specific for α2,3-linked sialic acids and produces 2,7-anhydro-Neu5Ac (Li et al. 1990; Tailford et al. 2015). Sialidases are distributed in vertebrates widely, and they can also be found in microorganisms including viruses, bacteria, fungi, mycoplasma, and protozoa. Sialidase activity has been shown in higher organisms and in a wide variety of mammalian cells and tissues for a long time. In early years, it was not clear whether the activities originated from the same or different types of sialidase due to the molecular instability and low levels of expression. After biochemical isolation and characterization of sialidases from rat tissues, evidence was provided, and four types of sialidases were shown that differ in their subcellular localization and enzymatic properties.

To date, there are four types of mammalian sialidases that have been identified and characterized as NEU1, NEU2, NEU3, and NEU4 (Taeko 2010). Today, it is known that NEU1, NEU2, and NEU3 are localized predominantly in the lysosomes, cytosol, and plasma membranes, respectively, whereas NEU4 is found in lysosomes or in mitochondria and endoplasmic reticulum (Miyagi and Yamaguchi 2012). Major functions of the four groups of sialidases are given in Fig. 3 (Miyagi and Yamaguchi 2012). The presence of NEU1 has recently been reported in the outer and NEU3 in the inner membrane of the nuclear envelope (Wang et al. 2009). NEU2 has been detected in cytosol and in the nucleoplasm of rat muscle fibers (Akita et al. 1997).

In humans, the identity of NEU1 to other sialidases is low, but NEU2, NEU3, and NEU4 show higher homology to each other, and their substrate specificities are also different. For example, NEU1 hardly hydrolyzes gangliosides, and NEU3 acts more on gangliosides but not on glycoproteins, whereas NEU4 acts on mucin (Seyrantepe et al. 2003; Yamaguchi et al. 2005). They also have different glycosidic linkage

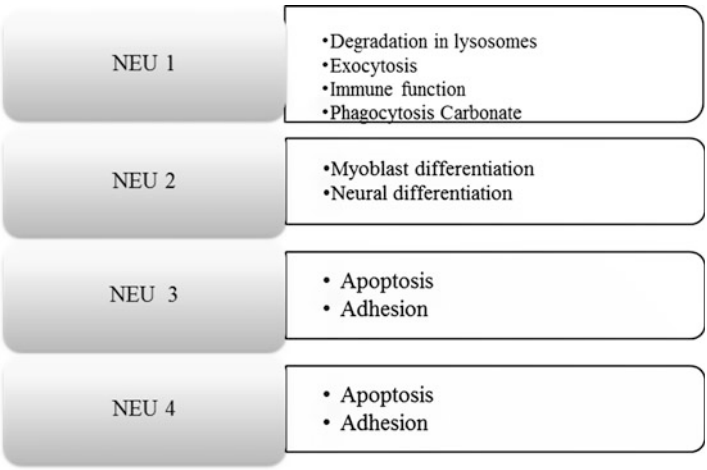

Fig. 3 Major functions of sialidases. Major functions of four sialidases groups are listed. *NEU 1* neuraminidase (sialidase) 1, *NEU 2* neuraminidase (Sialidase) 2, *NEU 3* neuraminidase (sialidase) 3, *NEU 4* neuraminidase (sialidase) 4

specificity, like oligosaccharide substrates possessing the α2,3 sialyl linkage that are hydrolyzed faster than those with α2,6 and α2,8 linkages by NEU1 (Miyagi and Tsuiki 1984).

In recent years, sialidases have been suggested to be candidate therapeutic agents for some diseases such as spinal cord injury, and for future approaches and therapeutic purposes, modulation of sialidase expression can be maintained by the appropriate use of recombinant sialidases technology (Miyagi and Yamaguchi 2012).

Sialidases in Liver Disease

It was reported that a major fraction of sialidase with activity toward ganglioside substrate was localized in the plasma membrane of the liver cell and that ganglioside substrate was also localized in this cellular structure (Schengrund et al. 1972).

In 1981, intracellular α-L-fucosidase and hexosaminidase have been reported to show similar isoelectric focusing patterns in control, cystic fibrosis, and neuraminidase (sialidase)-deficient fibroblasts. They were unaffected by neuraminidase treatment. Extracellular hexosaminidases A and B were found to be sensitive to neuraminidase for the cell types where cystic fibrosis extracellular α-L-fucosidase and hexosaminidase acted as for control fibroblasts (Butterworth and Priestman 1981). Sialidase activity of peripheral mononuclear cells, which are mostly lymphocytes prepared from patients with alcoholic liver disease, was found to be decreased or not increased in 50% of the cases (Matsuzaki et al. 1987).

Decreased Neu1 expression has been shown in various cancers, and there is an inverse relationship between NEU1 expression level and metastatic ability, also evident with the NEU1 reduction which occurs in rat 3Y1 fibroblasts after src-transformation. Moreover, a more severe decrease in sialidase activity and acquired higher lung metastatic potential was evident after v-fos transfer to these transformed cells (Taeko 2010). The in vivo liver metastatic potential significantly reduced after the injection of NEU1-overexpressing cells transsplenically into mice. Integrin β4 has been found to be one of the target molecules for NEU1 which underwent desialylation and decreased phosphorylation (Uemura et al. 2009).

NEU3 which was originally described as a plasma membrane ganglioside sialidase is a peripheral or extrinsic membrane-associated enzyme with the ability to act on gangliosides located on the same membrane or on the membrane of adjacent cells that also play a major role in cell-cell interactions (Fanzani et al. 2012). Liver NEU3 overexpression has been shown to positively improve insulin sensitivity and glucose tolerance in C57BL/6 and insulin-resistant mice by increased deposition of glycogen and triglycerides (Yoshizumi et al. 2007). This suggested that the effects of NEU3 on insulin responsiveness may be different between the skeletal muscle and liver depending on the tissue-specific pattern of gangliosides (Fanzani et al. 2012).

Neuraminidase inhibitors are another important subject related to sialidases. They have been widely used in Japan since 2001 with some adverse effects of their use

being reported. A case of generalized rash and erythema toxicum after treatment with the neuraminidase inhibitors administered prophylactically to prevent influenza infection in two patients with hepatoma associated with liver cirrhosis has been reported (Kaji et al. 2005).

The Interaction of Sialyltransferases, Sialidases, and Sialic Acids in Liver Diseases

Sialic acid has been shown to be increased in many pathological states where the pathology is due to tissue destruction, tissue proliferation, depolymerization, or inflammation. Accordingly, in liver cirrhosis, liver cancer, viral hepatitis, fatty liver, and hepatoma, abnormal sialic acid levels and alterations in sialyltransferases and sialidases enzymes have been reported as mentioned in previous sections. Variations in serum levels of sialyltransferases, sialidases, and sialic acids in these diseases are important diagnostic and prognostic tools. A large number of glycoproteins are secreted into the circulation by the liver, and they are sialylated on the termini of their glycans. The addition of these sialic acids are necessary for the survival of the serum proteins, and their removal can result in rapid clearance mediated by hepatic receptors that recognize the underlying sugar chain. Recently, the classic hepatic asialoglycoprotein "Ashwell receptor" has been shown to serve to reduce the level of coagulation determinants, like platelets and Von Willebrand factor (vWF), that have been desialylated by a sialidase released during sepsis with organisms like pneumococcus. Ashwell receptor rapidly clears glycoproteins bearing glycan ligands including galactose and *N*-acetylgalactosamine from circulation. This asialoglycoprotein receptor activity is suggested to be a key factor in the development and use of glycoprotein pharmaceuticals. The Ashwell receptor regulates vWF homeostasis and is responsible for thrombocytopenia during systemic *Streptococcus pneumoniae* infection through elimination of platelets desialylated by the bacterium's sialidase enzyme. Therefore, this clearance process might serve to avoid excessive intravascular coagulation and death and protect the organism (Grewal et al. 2008; Varki 2008).

Applications of Sialyltransferases, Sialidases, and Sialic Acids to Biomarker Discovery in Liver Diseases

The changes in glycosylation and sialylation of proteins and lipids play an important role in the pathogenesis and progression of liver diseases. Altered sialic acid expression is reported in many pathological states which can be detected in histological sections by using plant lectins or antibodies to detect specific sialylated glycans (Wearne et al. 2006). Sialic acid measurements of body fluids are used to predict disease risk, and in many studies, total sialic acid levels were measured to predict the risk of various diseases although the logic by which such measurements are of prognostic value is largely unknown. Their secretion might be increased as an

indication of an "acute phase response" (Varki 2008). The linking of sialic acid levels and sialylation of lipids and proteins in liver diseases can be detected in the serum as markers of liver disease progression. For instance, loss of sialylation on serum transferrin is used as a screening test both for chronic alcohol consumption and for congenital disorders of glycosylation (Varki 2008). On the other hand, sialidases can affect a number of different signaling pathways by modifying the cell content of gangliosides and sialylated receptorial and non-receptorial proteins although there are many things that need to be evaluated about sialidases in various physiological and pathological conditions of the liver. Proteomic techniques will produce a more complete characterization of sialidase substrates in order to fully understand their role. As a conclusion, a more detailed understanding of the role of sialic acid, sialidases, and sialyltransferases in liver diseases may significantly contribute to open new exciting frontiers of basic and therapeutic exploration.

Potential Applications to Prognosis, Other Diseases, or Conditions

Alterations in sialic acid levels are related to cancer, and analysis of the enzymes involved in addition of terminal sugars can give useful information to the understanding of the mechanism of elevations in sialic acid values during malignancy. Accordingly, increased circulatory sialyltransferase activities have been reported in various types of cancer. Therefore, determination of sialyltransferase in tumor tissue and sera of cancer patients may be useful for tumor detection and monitoring (Raval et al. 2004). On the other hand, sialidases play important roles in the cell by regulating the content of cellular sialic acid through the removal of sialic acid residues from glycoproteins and glycolipids. The altered and aberrant sialylation is related to malignant properties like invasiveness and metastatic potential. Moreover, in some cancers, in order to discriminate cancerous from noncancerous tissues and to determine the pathological stage, estimation of the mRNA levels of sialidases has been suggested. Additionally, immunohistochemical evaluation of cancer tissues using the antibody against the plasma membrane sialidase has been shown to be useful for clinical diagnosis (Miyagi et al. 2004, 2008). Therefore sialidases are considered as potential targets for cancer diagnosis and therapy.

Summary Points

- This chapter focuses on the interaction of sialyltransferases, sialidases, and sialic acids in liver diseases.
- Sialic acids are a different family of extraordinary monosaccharides sharing nine-carbon backbone that are thoroughly expressed on all cell surfaces of vertebrates and "higher" invertebrates and also on specific bacteria that interact with vertebrates.

- Sialic acid is being studied in liver diseases since the last two decades, and changes in the carbohydrate content of plasma glycoproteins have been shown in patients with different liver diseases.
- Sialic acid in the human serum has been shown to be higher in a number of pathological states where the pathology is either due to tissue destruction, tissue proliferation, depolymerization, or inflammation.
- Sialyltransferase group of enzymes catalyzes the transfer of the activated form of sialic acid onto the acceptor sugar. They catalyze the formation of different linkages (α2,3, α2,6, and α2,8), and they differ in their acceptor specificities.
- Sialidases (EC 3.2.1.18, also called neuraminidases) are glycosidases that catalyze the removal of sialic acid residues linked α-glycosidically from carbohydrate groups of glycoproteins and glycolipids. Based on their substrate specificity and catalytic mechanism, they are separated into three different classes as hydrolytic sialidases, trans-sialidases, and IT-sialidases.
- Altered sialic acid levels, sialyltransferase, and sialidase activities and expressions have been reported in many pathological states related to the liver.
- The interaction of sialic acid, sialyltransferase, and sialidase contributes to open new exciting frontiers of basic and therapeutic exploration in liver diseases.

References

Akita H, Miyagi T, Hata K, Kagayama M. Immunohistochemical evidence for the existence of rat cytosolic sialidase in rat skeletal muscles. Histochem Cell Biol. 1997;107:495–503.

Alturfan AA, Aytaç E, Emekli-Alturfan E, Yarat A, Sarıbeyoğlu K, Pekmezci S, Seymen O. Serum total sialic acid as a novel complementary candidate marker of hepatic damage in obstructive jaundice. Ann Clin Lab Sci. 2014;44:56–61.

Arif S, Haq N, Hanif R, Khan AS, Rehman J, Mufti TA. Variations of serum sialic acid level in liver cirrhosis. J Ayub Med Coll Abbottabad. 2005;17:54–57.

Breen KC, Potratz A, Georgopoulou N, Sandhoff K. The generation and characterization of a rat neural cell line overexpressing the a2,6(N) sialyltransferase. Glycoconj J. 1998;15:199–202.

Butterworth J, Priestman D. Susceptibility to neuraminidase of alpha-L-fucosidase and *N*-acetyl-beta-D-glucosaminidase of cystic fibrosis, I-cell and neuraminidase-deficient fibroblasts. Clin Chim Acta. 1981;5:319–26.

Byrne B, Donohoe GG, O'Kennedy R. Sialic acids: carbohydrate moieties that influence the biological and physical properties of biopharmaceutical proteins and living cells. Drug Discov Today Ther Strateg. 2007;12:319–26.

Cao Y, Merling A, Crocker PR, Keller R, Schwartz-Albiez R. Differential expression of beta-galactoside alpha 2,6 sialyltransferase and sialoglycans in normal and cirrhotic liver and hepatocellular carcinoma. Lab Invest. 2002;82:1515–24.

Carlin AF, Uchiyama S, Chang YC, Lewis AL, Nizet V, Varki A. Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil siglec-9 and dampen the innate immune response. Blood. 2009;113:3333–6.

Carlson J. α-antitrypsin and other acute phase reactants in liver-disease. Acta Med Scand. 1980;207:79–80.

Chrostek L, Supronowicz L, Panasiuk A, Cylwik B, Gruszewska E, Szmitkowski M. Serum sialic acids levels according to the severity of liver cirrhosis. J Clin Lab Anal. 2014;28:465–8.

Fanzani A, Zanola A, Faggi F, Papini N, Venerando B, Tettamanti G, Sampaolesi M, Monti E. Implications for the mammalian sialidases in the physiopathology of skeletal muscle. Skelet Muscle. 2012;1:23–4.

Gong M, Castillo L, Redman RS, Garige M, Hirsch K, Azuine M, Amdur RL, Seth D, Haber PS, Lakshman MR. Down-regulation of liver Galbeta1, 4GlcNAc alpha2, 6-sialyltransferase gene by ethanol significantly correlates with alcoholic steatosis in humans. Metab Clin Exp. 2008;57:1663–8.

Grewal PK, Uchiyama S, Ditto D, Varki N, Le DT, Nizet V, Marth JD. The Ashwell receptor mitigates the lethal coagulopathy of sepsis. Nat Med. 2008;14:648–55.

Gruszewska E, Cylwik B, Panasiuk A, Szmitkowski M, Flisiak R, Chrostek L. Total and free serum sialic acid concentration in liver diseases. Biomed Res Int. 2014. 10.1155/2014/876096. Accessed 18 May 2014.

Gruszewska E, Chrostek L. Biomarkers in Liver Disease, Biomarkers in Disease: Methods, Discoveries and Applications. In: Preedy VR, Patel VB, editors. Serum sialic acid as a biomarker in liver disease Serum sialic acid as a biomarker in liver disease. 1st ed. Springer; 2016. in press. DOI 10.1007/978-94-007-7742-2_19-1

Harduin-Lepers A, Vallejo-Ruiz V, Krzewinski-Recchi MA, Samyn-Petit B, Julien S, Delannoy P. The human sialyltransferase family. Biochimie. 2001;83:727–37.

Harduin-Lepers A, Mollicone R, Delannoy P, Oriol R. The animal sialyltransferases and sialyltransferase-related genes: a phylogenetic approach. Glycobiology. 2005;15:805–17.

Henderson M, Kessel D. Alterations in plasma sialyltransferase levels in patients with neoplastic disease. Cancer. 1977;39:1129–34.

Kaji M, Fukuda T, Tanaka M, Aizawa H. A side effect of neuraminidase inhibitor in a patient with liver cirrhosis. J Infect Chemother. 2005;11:41–3.

Kaniak J, Mejbaum KBW, Jelewska KZ, Kudreweiz HZ, Kowal GZ. Sialic acid contents of glycoproteins and seromucoid in liver diseases. Pol Med J. 1980;1:1076–81.

Kuhlenschmidt MS, Peters SP, Pinkard OD, Glew RH, Sharp H. Asialoglycoprotein sialic acid transferase activity in liver and serum of patients with juvenile hepatic cirrhosis and alpha-1-antitrypsin deficiency. Biochim Biophys Acta. 1976;8:359–73.

Li YT, Nakagawa H, Ross SA, Hansson GC, Li SC. A novel sialidase which releases 2,7-anhydro-alpha-*N*-acetylneuraminic acid from sialoglycoconjugates. J Biol Chem. 1990;265:21629–33.

Lindberg G, Iso H, Rastam L, Lundblad A, Folsom AR. Serum sialic acid and its correlates in community samples from Akita, Japan and Minneapolis. Int J Epidemiol. 1997;26:58–63.

Lu J, Gu J. Significance of β-galactoside α2,6 sialyltransferase 1 in cancers. Molecules. 2015;20:7509–27.

Martinez J, Palascak JE, Kwasniak D. Abnormal sialic acid content of dysfibrinogenemia associated with liver disease. J Clin Invest. 1978;61:535–8.

Matsuzaki S, Itakura M, Iwamura K, Kamiguchi H. Serum sialic acid levels in liver cirrhosis and liver cancer. Nippon Shonika Gakkai Zasshi. 1981;78:2395–401.

Matsuzaki S, Itakura M, Kadosaka T, Kamiguchi H, Yamamura M, Katsunuma T. Effect of ethanol on sialidase activity of peripheral lymphocytes. Alcohol Alcohol Suppl. 1987;1:509–11.

Miyagi T, Tsuiki S. Rat-liver lysosomal sialidase. Solubilization, substrate specificity and comparison with the cytosolic sialidase. Eur J Biochem. 1984;141:75–81.

Miyagi T, Yamaguchi K. Mammalian sialidases: physiological and pathological roles in cellular functions. Glycobiology. 2012;22:880–96.

Miyagi T, Wada T, Yamuguchi K, Hata K. Sialidase and malignancy. Glycoconj J. 2004;20:189–98.

Miyagi T, Wada T, Yamaguchi K, Shiozaki K, Sato I, Kakugawa Y, Yamanami H, Fujiya T. Human sialidase as a cancer marker. Proteomics. 2008;8:3303–11.

Mühlenhoff M. Polysialic acid: three-dimensional structure, biosynthesis and function. Curr Opin Struct Biol. 1998;8:558–64.

Narvaiza MJ, Fernandez J, Cuesta B, Paramo JA, Rocha E. Role of sialic acid in acquired dysfibrinogenemia associated with liver cirrhosis. Ric Clin Lab. 1986;16:563–8.

O'Kennedy R, Berns G, Moran E, Smyth H, Carroll K, Thornes RD. A critical analysis of the use of sialic acid determination in the diagnosis of malignancy. Cancer Lett. 1991;58:91–100.
Okude M, Yamanka A, Moriomoto Y, Akihama S. Sialic acid in fibrinogen: effects of sialic acid on fibrinogen-fibrin conversion by thrombin and properties of asialofibrin clot. Biol Pharm Bull. 1993;16:448–52.
Okude M, Yamanaka A, Akihama S. The effects of pH on the generation of turbidity and elasticity associated with fibrinogen fibrin conversion by thrombin are remarkably influenced by sialic acid in fibrinogen. Biol Pharm Bull. 1995;18:203–7.
Pinheiro VAC, Santos SFC, Bressan J. Hepatic inflammatory biomarkers and its link with obesity and chronic diseases. Nutr Hosp. 2015;1:1947–56.
Raval GN, Parekh LJ, Patel DD, Jha FP, Sainger RN, Patel PS. Clinical usefulness of alterations in sialic acid, sialyltransferase and sialoproteins in breast cancer. Indian J Clin Biochem. 2004;19:60–71.
Schengrund CL, Jensen DS, Rosenberg A. Localization of sialidase in the plasma membrane of rat liver cells. J Biol Chem. 1972;10:2742–6.
Seyrantepe V, Poupetova H, Froissart R, Zabot MT, Maire I, Pshezhetsky AV. Molecular pathology of NEU1 gene in sialidosis. Hum Mutat. 2003;22:343–52.
Taeko M. Mammalian sialidases and their functions. Trends Glycosci Glcy. 2010;22:162–72.
Tailford LE, Owen CD, Walshaw J, Crost EH, Hardy-Goddard J, Gall GL, de Vos WM, Taylor GL, Juge N. Discovery of intramolecular trans-sialidases in human gut microbiota suggests novel mechanisms of mucosal adaptation. Nat Commun. 2015;6:7624–5.
Traving C, Schauer R. Structure, function and metabolism of sialic acids. Cell Mol Life Sci. 1998;54:1330–49.
Uemura T, Shiozaki K, Yamaguchi K, Miyazaki S, Satomi S, Kato K, Sakuraba H, Miyagi T. Contribution of sialidase NEU1 to suppression of metastasis of human colon cancer cells through desialylation of integrin beta4. Oncogene. 2009;28:1218–29.
Varki A. Sialic acids in human health and disease. Trends Mol Med. 2008;14:351–60.
Varki A, Gagneux P. Multifarious roles of sialic acids in immunity. Ann NY Acad Sci. 2012;1253:16–36.
Wang J, Wu G, Miyagi T, Lu ZH, Ledeen RW. Sialidase occurs in both membranes of the nuclear envelope and hydrolyzes endogenous GD1a. J Neurochem. 2009;111:547–54.
Wearne KA, Winter HC, O'Shea K, Goldstein IJ. Use of lectins for probing differentiated human embryonic stem cells for carbohydrates. Glycobiology. 2006;16:981–90.
Werle M, Bernkop-Schnürch A. Strategies to improve plasma half life time of peptide and protein drugs. Amino Acids. 2006;30:351–67.
Yamaguchi K, Hata K, Koseki K, Shiozaki K, Akita H, Wada T, Moriya S, Miyagi T. Evidence for mitochondrial localization of a novel human sialidase (NEU4). Biochem J. 2005;390:85–93.
Yoshizumi S, Suzuki S, Hirai M, Hinokio Y, Yamada T, Tsunoda U, Aburatani H, Yamaguchi K, Miyagi T, Oka Y. Increased hepatic expression of ganglioside-specific sialidase, NEU3, improves insulin sensitivity and glucose tolerance in mice. Metabolism. 2007;56:420–9.

Part II

Body Fluids, Tissue, and Specific Biomarkers

Biomarkers in Focus: Alanine Aminotransferase

12

Guido Engelmann

Contents

Abstract

Alanine aminotransferase (ALAT) serves as a surrogate marker for liver disease. It originates from the core of the hepatocyte. In very small amounts, it might also be detected in muscle cells. In contrast to other markers of liver cell integrity like ASAT or Lactate Dehydrogenase, ALAT is highly specific for liver diseases. The sensitivity of elevated ALAT for hepatopathies is as high as 84% in large studies. The life span of a human liver cell is between 1 and 8 months. Due to the fact that liver cells are permanently replaced, there is always some ALAT detectable in the bloodstream. An elevation of ALAT of 15 times above ULN has a 100% sensitivity for a liver disease.

G. Engelmann (✉)
Department of Pediatrics, Lukas Hospital, Neuss, Germany
e-mail: guido.engelmann@me.com; Engelmann@lukasneuss.de

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_31

Keywords
ALAT • Aminotransferase • Hepatocyte integrity

List of Abbreviations

ALD	Alcoholic liver disease
ALAT	Alanine aminotransferase
ALF	Acute liver failure
AMA	Antimitochondrial antibody
APRI	Aspartate aminotransferase to platelet ratio index
ASAT	Aspartate aminotransferase
BMI	Body mass index
GGT	Gamma glutamyltransferase
INR	International normalized ratio
LDH	Lactate dehydrogenase
NAD	Nicotinamide adenine dinucleotide
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
OTCD	Ornithine transcarbamylase deficiency
PBS	Primary biliary cirrhosis
PSC	Primary sclerosing cholangitis
UDC	Ursodeoxycholic acid
ULN	Upper limit of normal

Key Facts of Alanine Aminotransferases

- Originating almost exclusively from the liver cell
- Catalyze reactions between amino acids and keto acids
- Part of protein synthesis
- Stable values in serum and in repeated measurements
- No international normalized limits of normal

Definition of Words and Terms

Acute liver failure	Liver disease with laboratory evidence of liver dysfunction and onset of hepatic encephalopathy (HE) within 8 weeks of the onset of jaundice.
Alcoholic liver disease	Liver disease related to alcohol consumption with either fatty liver, alcoholic hepatitis or cirrhosis.
Nonalcoholic fatty liver disease	Fatty accumulation within the liver cells without signs of inflammation in the absence of significant consumption of alcohol.

Nonalcoholic steatohepatitis	Fatty accumulation within the liver cells with signs of inflammation in the absence of significant consumption of alcohol.
Primary biliary cirrhosis	A chronic granulomatous inflammation of the small bile ducts most likely due to autoimmune activity.
Primary sclerosing cholangitis	A chronic disease of large and small bile ducts with stenosing of bile ducts, fibroses of the liver, and signs of inflammation.
Upper limit of normal	The ULN of an enzyme in the blood is defined as mean enzyme level measured in a healthy population plus 2 standard deviations (SDs).
Ursodeoxycholic acid	A hydrophilic bile acid that naturally occurs in the bile of Chinese brown bear and in small amounts in the bile of humans.

Introduction

"Glutamic-pyruvic transaminase" was first described in 1955 as a possible marker of cardiac infarction (Poynard et al. 2004; Henley and Pollard 1955). Later, the name was changed to alanine aminotransferase because enzymes that catalyze a transamination are generally called aminotransferases.

Aminotransferases are widely used as a screening tool for the detection of liver, heart, and muscle diseases and of hemolysis. They can easily be measured in serum specimen. In this review, the focus is on ALAT, the more liver-specific aminotransferase.

That is either calculating two ways. Either 2 standard deviations (SD) above mean in a healthy population or performing a ROC analyses. Both methods are used in patientgroups with a defined disease (here hepatitis C) and in a (presumed) liver healthy population. Presumably healthy population was defined in a large study as people with ". . .negative HCV RNA and hepatitis B surface antigen, low alcohol consumption, no evidence of diabetes, and normal body mass index and waist circumference") (Ruhl and Everhart 2012). In a study by Ruhl and colleagues the ROC method has led to a ULN of ALAT of 24 IU/L (70% specificity) for men and 18 IU/L (63% specificity) for women. As these ULNs are extremely low, using them in clinical practice would produce a very high percentage of healthy persons with elevated ALAT (around 30%).

It seems more useful to take the 2 SDs above mean values for clinical practice. The higher 2 SDs above mean values are much more common.

Introduction

Due to its nature as an organ-specific enzyme, ALAT serves as a screening marker for liver cell integrity. ALAT is localized almost exclusively in hepatocytes and in very small amounts in kidney, heart, and muscle cells. ALAT is localized in the

cytoplasm of the hepatocyte in a concentration that is around 3000 times higher than in the blood stream (Murawaki et al. 1997; Kim et al. 2008; Iredale et al. 1996). In diseases of the liver, either permeability of hepatocyte membrane increases followed by a rise of serum aminotransferases or the membrane is disintegrated (toxic, trauma, vascular) and the aminotransferases are set free immediately, reaching extremely high levels more than 25 times the ULN. In cases where the hepatocytes become completely necrotic (fulminant hepatic failure) and there is hardly any vital hepatocyte left, ALAT finally decreases, while parameters of detoxification (like bilirubin or NH_3) and parameters of liver synthesis (INR or cholesterol) quickly increase.

In healthy persons, there is always some ALAT detectable in the bloodstream. This is due to the fact that liver cells are permanently eliminated and replaced by new cells. The life span of an average human liver cell is 1 to 8 months. ALAT is cleared from the blood stream in hepatic sinusoidal cells. ALAT clearance results in a half-life time of ALAT of 47 $\pm$ 10 h compared to 17 $\pm$ 5 h for ASAT (Stetler-Stevenson et al. 1997). ALAT elevation is infrequently found in healthy volunteers. A population-based study detected an ALAT elevation in healthy persons in 8.9% of cases (Pratt and Kaplan 2000). In its first description, ALAT was evaluated as a marker of myocardial infarction and served as the first biological marker for cardiac infarction long before creatine kinase or troponin T and the more modern markers were discovered (Lewandrowski et al. 2002).

ALAT activity is measured in serum. The most widely used method today is based on the enzymatic activity of ALAT. It catalyzes the formation of pyruvic acid, finally leading to oxidation of NAD to NADH. This oxidation can be measured spectrophotometrically. There are numerous essays to measure the enzyme activity, each with a specific upper limit of normal (ULN). Therefore, no international available ULN exists and ULN is always depending on the recommendations of the manufacturer of the specific essay. Basically, ULN is defined as mean aminotransferase level in a presumed healthy population plus 2 standard deviations (Siest et al. 1975, Table 4). In a population-based study in 12,682 volunteers from the region of Nancy (France) in 1975 ULN for aminotransferases was defined for the first time. Siest and coworkers showed a day-to-day variability (Table 2), an age (negative correlation) and sex (ALAT in men > ALAT in women, Table 3) dependence, and intraday differences with highest levels in the late afternoon. The normal values and the variability of values are shown in Table 1 (Yoshiji et al. 2000; Siest et al. 1975).

ALAT activity shows its highest levels in the afternoon with up to 45% difference to values measured in the morning. The day-to-day variability may reach 10–30% (Nie et al. 2001; Fraser 1992) (Table 2).

ALAT serves as a widely available, stable, and low-cost surrogate marker for liver disease in general. Its prognostic value over all for liver diseases is high. The specificity is 83% with a sensitivity of 98% toward healthy patients (84% toward patients with other diseases). In more than 60% of patients in a Swedish study with mild to moderate elevation of ALAT for more than 6 months, a liver disease was detected (mainly nonalcoholic fatty liver disease (NAFLD), chronic viral hepatitis, and alcohol consumption).

Table 1 Aminotransferase activities (U/liter) as a function of sex and standard meal intake

	Alanine aminotransferase			
	Males		Females	
Standard meal intake	Before	After	Before	After
Age				
6–10 years	18.4	17.8	18.2	17.5
10–15 years	17.9	19.0	17.3	17.1
15–20 years	19.1	19.4	16.4	16.4
20–30 years	23.7	23.9	17.5	17.3
30–40 years	27.5	28.1	18.1	18.5
40–50 years	29.3	28.2	18.6	19.6
50–60 years	28.2	27.1	21.5	21
60 years	23.6	22.2	20.3	20.2

Table 2 Analytical variations, within day and from day to day (Nie et al. 2001; Fraser 1992)

	Within day			Day to day		
	I	II	III	March	May	July
No. of essays	30	30	30	20	20	20
	Alanine aminotransferase activity					
Mean (U/liter)	17.3	19.2	20.6	9.3	9.3	9.0
Standard deviation (SD)	0.7	0.6	0.4	0.3	0.4	0.5
Variation coefficient (CV), %	4.3	3.2	2.0	3.4	4.8	6.4

Table 3 Correlation of ALAT level and gender (Poynard et al. 2004; Boeker et al. 2002; Henley and Pollard 1955; Kim et al. 2008)

	Women	Men
Age	+	–
BMI	++	++
Triglyceride	++	++
Cholesterol		+
Alcohol consumption		+
Smoking		–
Physical activity		–
Glucose	+	
Oral contraception	–	

Table 4 Normal values of ALT in percentiles from Siest et al. (1975)

Percentile limits for aspartate aminotransferase activities					
U/l	2.5	50	90	95	97.5
Males	13.4	26.3	55.8	60.7	77
Females	10.7	19.1	33.6	38	51

ALAT can be temporarily elevated in many diseases without relevant liver damage. Therefore detection of an elevation of ALAT of less than five times the ULN at a single time point should lead to a repeated control of liver enzymes, before

starting a sophisticated diagnostic evaluation (Ruhl and Everhart 2012; Ferraioli et al. 2012; Kim et al. 2008).

ALAT is widely used in clinical practice as a marker for liver health. ALAT within the normal range is presumed to be a sign of a healthy liver. This holds true for most patients, but the specificity of 83% and a negative predictive value of 98% demonstrate that patients with normal ALAT may have an underlying liver disease with a very slow progress. In a series of 222 patients with histologically proven NAFLD, 37% with normal ALAT showed advanced fibrosis or NASH. Therefore, in liver cirrhosis and fatty liver disease, aminotransferases may appear within the normal range despite a relevant hepatopathy. Thus, if cirrhosis or NASH is suspected, other markers of changes in liver structure (histology or noninvasive measurement of liver fibrosis) are needed.

Liver fibroses and liver cirrhosis are continuing aspects of dynamic changes in liver structure forced by liver diseases (for review see Ruhl and Everhart 2012; Squires et al. 2006; Schuppan and Kim 2013). These structural changes usually appear slowly within years. Liver cirrhosis represents the end-stage of fibrotic liver diseases. The development from liver fibroses to cirrhosis may be preventable, if fibroses is detected early in the course and if an intervention is possible as in infections with hepatitis B or hepatitis C (Murawaki et al. 1997; Dhawan et al. 2004; Kim et al. 2008; Manns et al. 2014; Iredale et al. 1996) or in Wilson's disease. Today the gold standard for diagnosing structural changes of the liver is histological evaluation of a liver specimen by an experienced pathologist. This specimen should have a length of at least 10 mm and a width of at least 1 mm (>18 Gauge needle) (Stetler-Stevenson et al. 1997; Kelly and McKiernan 1998; Colloredo et al. 2003). Several histological scoring systems have been established for grading (necroinflammatory activity) and staging (fibrosis) in patients with structural liver damage (Pratt and Kaplan 2000; Bhaduri and Mieli-Vergani 1996; Cholongitas et al. 2006). The Desmet score (Yoshiji et al. 2000; Kim et al. 2008; Siest et al. 1975; Desmet et al. 1994) is evaluated in adult hepatitis C patients and METAVIR (Nie et al. 2001; Sokol 2002; Fraser 1992; Bedossa and Poynard 1996) and Ishak score (Tanner 2002; Ishak et al. 1995) in chronic viral hepatitis (b and c). The SSS score of Chevallier (American Gastroenterological Association 2002; Chevallier et al. 1994) has been developed to quantify fibroses irrespective of the underlying disease. But liver biopsy has technical limitations. There is a small risk of clinical relevant bleeding (0.3%) and mortality of 0.04–0.07% in large series (Neuschwander-Tetri and Caldwell 2003; Atwell et al. 2010). In a pediatric series, there were major complications in 1.5% and minor complications in 25% of 275 liver biopsies (Angulo 2002; Westheim et al. 2012; Molleston et al. 2002). Another drawback is the nature of liver disease and the sample size (Sanyal et al. 2001; Maharaj et al. 1986). With a single liver biopsy, there is a 20–30% chance of missing the relevant area of interest, thus underestimating liver diseases (Poynard et al. 2004).

Reliable noninvasive methods to diagnose and quantify liver fibrosis have been developed recently. Serum biomarkers have been investigated in several studies demonstrating promising results in defined diseases. The expression of TIMP is

elevated in human patients with liver fibrosis (Murawaki et al. 1997; Iredale et al. 1996). An important endogenous inhibitor of interstitial collagenase is TIMP 1 (Stetler-Stevenson et al. 1997). Further studies showed that high TIMP1 promotes liver fibrosis in mice (Yoshiji et al. 2000) and inhibition of TIMP1 expression weakens the process of liver fibrosis (Nie et al. 2001). In a chronic hepatitis C patient, a strong correlation between TIMP1 serum level and liver fibrosis was shown (Boeker et al. 2002).

Besides serological tests like TIMP1 or aspartate aminotransferase to platelet ratio index (APRI) developed by Wai et al. (2003), ultrasound-based systems that directly measure or indirectly determine liver stiffness have been introduced in clinical routine today. Transient elastography (TE) is a technique based on the measurement of the velocity of a shear wave that is induced to the liver by a mechanical impulse. This velocity of the shear wave reflects the stiffness of the liver. Stiffness primarily depends on the amount of extracellular matrix in the liver. Therefore liver stiffness measurement (LSM) in kilopascal (kPa) is equivalent with fibrosis.

Ultrasound elastography is another method based on ultrasound. Several techniques have been developed recently. Real-time tissue elastography (rTE) (Ferraioli et al. 2012) which is based on the measurement of tissue motions that are induced by heart beat or respiration is widely used.

Potential Applications to Prognosis, Other Diseases, or Conditions

Acute Liver Failure

Acute liver failure (ALF) is defined as hepatic encephalopathy in combination with an elevated prothrombin time/international normalized ratio (INR) in patients with acute liver injury. ALF in children is defined as INR above 1.5 and hepatic encephalopathy or an INR above 2 in absents of encephalopathy due to a liver disease of less than 8 weeks duration (Squires et al. 2006). Patients usually present with jaundice, elevated aminotransferases, hypoglycemia, hyperammonemia, decrease of coagulation factors, spontaneous bleeding, hypoalbuminemia and ascites. Hepatic encephalopathy is infrequently found in children, especially in those under 2 years of age (Dhawan et al. 2004).

ALF is a life-threatening event with a high mortality. Viral infections, poisoning, and metabolic or autoimmune diseases are the main causes. Some inborn errors of metabolism have been described to cause ALF in infancy and childhood such as fatty acid oxidation defects (Kelly and McKiernan 1998), classical galactosemia, mitochondriopathies such as Alpers' syndrome or tyrosinemia type I. Ornithine transcarbamylase deficiency, a urea cycle disorder, is also known to cause ALF (Bhaduri and Mieli-Vergani 1996).

The distribution of ALAT (cytoplasm) and ASAT (mitochondria 70%, cytoplasm 30%) in the hepatocyte in combination with the different half-life time in serum leads

to a very fast elevation of ASAT within the first 24 h of injury followed by a fast increase of ALAT that exceeds ASAT at day 3 of injury. An elevation of ALAT above 15 × ULN (i.e., above 750 IU/ml) should lead to immediate diagnostic procedures focusing on acute liver failure, viral hepatitis, autoimmune hepatitis, vascular disorders, traumatic liver injury, and intoxication (Kim et al. 2008).

In many countries, "over-the-counter medications" and herbal preparations cause unexpected ALAT elevation. Especially in unexplained liver failure, it is necessary to search for drugs including herbal preparations.

Liver Fibroses and Cirrhosis

Liver fibrosis is the result of dynamic reactions of a healthy liver toward chronic cell injury (Sokol 2002). It can be observed in a large proportion of patients with chronic liver disease, regardless of its cause (Tanner 2002). Early treatment of the underlying disease may limit the progression of fibrosis. An early detection and treatment of fibrotic changes is important to avoid complications like portal hypertension with esophageal varices or ascites.

Aminotransferases poorly reflect the stage of liver fibrosis or cirrhosis. They may even be normal or only slightly elevated in fibrotic or cirrhotic livers. But ALAT may still be useful in screening for fibrosis. If it is normal, it may be used in combination with thrombocyte count to perform the aspartate aminotransferase to platelet ratio index (APRI) score. This index has been validated in patients with chronic liver diseases like hepatitis B or C. The negative predictive value of this test for fibrosis is 90% (APRI score <0.5) and reaches nearly 100% for cirrhosis if the APRI score is less than 1.

However, in patients with suspected fibrotic or cirrhotic changes of liver structure, the gold standard for diagnosis and follow-up of liver fibrosis or cirrhosis is the histologic examination of a liver specimen obtained by percutaneous liver biopsy. Liver biopsy is the method of choice in clarifying the etiology of hepatopathies. For follow-up, noninvasive methods like transient elastography may be useful.

NAFLD: NASH

NAFLD is defined as an accumulation of fat in the cytoplasm of the hepatocyte in absence of significant alcohol consumption. Histology is characterized by a macrovesicular hepatic steatosis (American Gastroenterological Association 2002). NASH is a liver disease in absence of significant alcohol consumption with accumulation of fat into the hepatocyte and signs of hepatic fibroses and/or inflammation (Neuschwander-Tetri and Caldwell 2003). NASH can progress to liver cirrhoses in up to 10% of adult patients and also in children, whereas NAFLD is believed to represent a benign form of fatty liver accumulation usually not progressing to cirrhosis (Angulo 2002; Molleston et al. 2002).

Obesity leads to accumulation of triacylglycerol in hepatocytes. This may cause either NAFLD or nonalcoholic steatohepatitis (NASH). There is strong evidence that in NAFLD patients insulin does not suppress lipolysis as strong as it does in non-obese patients (Sanyal et al. 2001) leading to an accelerated hepatic free fatty acid supply (Henley and Pollard 1955; Donnelly et al. 2005). This imbalance between uptake, synthesis, export, and oxidation of free fatty acids leads to accumulation of fat in the cytoplasm of the liver cells (Kim et al. 2008; Bugianesi et al. 2005). Fatty liver disease is often associated with the metabolic syndrome defined as hyperinsulinemia or a fasting blood glucose level >100 mg/dL or a blood glucose level >200 mg/dL 2 h after an oral glucose tolerance test plus either obesity, dyslipidemia, or hypertension (Alberti and Zimmet 1998; Nobili and Manco 2007). Furthermore patients with NAFLD carry a higher risk of developing cardiovascular diseases (Siest et al. 1975; Kelsey et al. 2014; Pacifico et al. 2008) or even hepatocellular carcinoma (Fraser 1992; Ip and Wang 2013). In the diagnostic approach of fatty liver disease, the first findings often are increased aminotransferase levels (Kim et al. 2008; Fishbein et al. 2005) although the sensitivity of serum ALAT is low. A recent study by Schwimmer et al. demonstrated a 57% sensitivity of ALAT for the detection of NAFLD or NASH (Kim et al. 2008; Schwimmer et al. 2013), and NASH and NAFLD also appear in patients with normal ASAT or ALAT (Schuppan and Kim 2013; Molleston et al. 2013). However, abnormal aminotransferases in daily practice serve as surrogate markers for fatty liver disease (Manns et al. 2014; Pearce et al. 2013) in most clinical settings, and a liver biopsy is usually performed only in obese patients with elevated liver enzymes, asking for NAFLD, NASH, autoimmune hepatitis, or Wilson's disease (Colloredo et al. 2003; Schwimmer et al. 2013).

ALAT elevations above ULN can be observed in fatty liver disease but do not predict the degree of inflammation in the liver. A nonalcoholic fatty liver disease (NAFLD) may show elevated liver enzymes but has no pathological relevance, while only 10% of these patients develop a NASH. NASH is a fatty liver disease with histological signs of inflammation and a tendency to liver structure changes (fibroses). About 10% of patients with NASH develop cirrhosis. The discrimination between NAFLD and NASH can be performed safe on the basis of a histological evaluation.

Wilson's Disease (For Review, See Cholongitas et al. 2006; Roberts et al. 2008)

Wilson's disease (WD) is a disease of copper transport. The defective copper transport leads to copper accumulation in several organs, especially in liver cells and in the brain. Hepatic Wilson's disease is diagnosed either due to mild to moderately elevated ALAT in routine examination, in acute liver failure, or in patients with specific neurologic symptoms (dysarthria, dystonia, tremor). Elevation of ALAT in patients with WD is mild to moderate, even in severe liver failure. Thus,

a combination of severe liver failure, mildly elevated ALAT, and Coombs negative hemolytic anemia is almost pathognomonic for an acute onset WD. Treatment of WD is based on chelation therapy. Binding of copper to potent copper chelators like D-penicillamine is the treatment of choice. In many patients, ALAT improves under this therapy, but ALAT neither is a good indicator for successful therapy nor for therapy adherence. About 30% of patients continue to have mildly elevated ALAT under successful chelating therapy. Therefore urine copper excretion is the better parameter for therapy adherence and timing of switch to maintenance therapy. D-Penicillamine therapy without side effects must never be stopped but can be switched to trientine if side effects appear.

Autoimmune Liver Disease (AIH, PSC, PBC)

Autoimmune Hepatitis AIH (For Review, See Desmet et al. 1994; Zachou et al. 2013)

Autoimmune hepatitis type I or type II (AIH) is a liver disease with specific antibody formation against surface antigens of hepatocytes. Its origin is unknown. AIH can be detected before symptoms appear by the presence of elevated aminotransferases, especially ALAT. If symptoms like fatigue or jaundice appear, ALAT is frequently elevated, but IgG and autoantibodies are more disease specific. In AIH, ALAT plays a specific role in monitoring of treatment. The goal of treatment is a decrease of immunoglobulines and of ALAT. If under prednisone or other immunosuppressants ALAT appears normal and IgG also appears normal, the patient is presumed to be in remission. Therefore frequent measurements of ALAT in patients with diagnosed AIH are the mainstay of therapy control.

Primary Sclerosing Cholangitis (For Review, See Bedossa and Poynard 1996; Lindor et al. 2015)

Primary sclerosing cholangitis (PSC) is an idiopathic chronic liver disease. It may appear in children and has a peak of appearance in young female adults. It appears almost exclusively in patients with inflammatory bowel disease. PSC causes severe liver damage by damaging the biliary tree causing obstructions, jaundice, and severe pruritus. An elevated ALAT can be observed as an early sign of liver cell damage even in asymptomatic patients. The ALAT elevation is usually moderate, except for those with an acute onset and liver failure. As cholestasis is the leading pathology and liver cell damage is thought to be secondary, ALAT is of no specific value in the management of PSC. Treatment with ursodeoxycholic acid (UDC) may lead to biochemical improvement. Whether this reflects an improvement of the liver cells is a matter of debate. Today there is hardly any other treatment of PSC than UDC and liver transplantation.

Primary Biliary Cirrhosis (PBC) (Ishak et al. 1995; Karlsen et al. 2014)

Primary biliary cirrhosis (PBC) is a cholestatic liver disease of the small bile ducts. The disease is the result of a T-cell-mediated attack to the small bile duct

epithelium. Ninety percent of patients are adult women. The disease hardly ever appears before the age of 20. In 50% of patients, the first signs are slightly elevated liver enzymes obtained for other reasons than liver disease and massively elevated alkaline phosphatase. The leading serologic marker of PBC is antimitochondrial antibody (AMA).

Treatment goal is biochemical response to UDC. Patients with an early and mild onset of disease and prompt initiation of UDC treatment and with a biochemical response seem to have a better prognosis than those without response.

Therefore, in treatment control, ALAT plays an important rule.

Alcoholic Liver Disease

Alcoholic liver disease (ALD) is the most common liver disease in the western world. It is frequently found in patients with heavy alcohol consumption for a long period of time (>100 g/day for 20 years). Most patients do not show an elevation of aminotransferases but of gamma glutamyltransferase (Chevallier et al. 1994; O'Shea et al. 2010). A total bilirubin/GGT ratio of >1 seems to be a good predictor of the 1-year mortality if an alcoholic cirrhosis already exists (Atwell et al. 2010; Poynard et al. 1984). In patients with aminotransferases elevation, ALAT is typically mildly elevated (<200 UI/ml) usually not exceeding 500 IU/ml, and ASAT is more elevated than ALAT (Westheim et al. 2012; O'Shea et al. 2010). This is demonstrated by a high De Ritis quote (ASAT/ALAT) of 2 or higher. This quote is almost exclusively found in alcoholic liver disease. In about 70% of patients, De Ritis quote may be significantly elevated especially in the absence of cirrhosis (Maharaj et al. 1986; Cohen and Kaplan 1979).

Other (Viral) Hepatites

ALAT levels in serum do not correlate with disease activity. End-stage liver cirrhosis may appear with only slightly elevated ALAT but severe liver failure like in acute Wilson's disease which may present with coagulopathy and severe cholestasis but only little elevation of liver enzymes between 100 and 500 UI/ml. On the other side, acute viral hepatitis or liver trauma may cause tremendous elevation of liver enzymes but complete restitutio ad integrum. Hepatitis due to an infection with hepatitis A, B, or C may be a reason for ALAT elevation. Other infectious agents causing an ALAT elevation are Epstein-Barr virus, cytomegalovirus, adenovirus, herpes virus (1 and 2), varicella virus, hepatitis E and D, HIV, parvovirus B19, and human herpes virus 6. But ALAT is not specific. In hepatitis A, it may rise and fall quickly, while in hepatitis B and C, it may be only slightly elevated. In the other viruses, ALAT may reach hardly any level depending on factors that are not understood today. Independent of the infectious agent, every infection may lead to a temporary liver enzyme elevation.

Summary Points

- Normal ALAT levels in serum represent the fact that liver cells are permanently degraded and rebuilt.
- Elevated ALAT levels are indicative for liver damage with a sensitivity of >90% and a specificity of 83%.
- Highly indicative for quick liver processes that lead to massive cell degradation.
- Not very indicative for slow processes in the liver like fibrosis/cirrhosis and fatty liver disease.
- ALAT is a surrogate marker for liver disease. A normal ALAT does not exclude liver disease.

References

Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med: J Br Diabet Assoc. 1998;15(7):539–53.

American Gastroenterological Association. American Gastroenterological Association medical position statement: nonalcoholic fatty liver disease. Gastroenterology. 2002;123(5):1702–4.

Angulo P. Nonalcoholic fatty liver disease. N Engl J Med. 2002;346(16):1221–31.

Atwell TD, et al. Incidence of bleeding after 15,181 percutaneous biopsies and the role of aspirin. AJR Am J Roentgenol. 2010;194(3):784–9.

Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology. 1996;24(2):289–93.

Bhaduri BR, Mieli-Vergani G. Fulminant hepatic failure: pediatric aspects. Semin Liver Dis. 1996;16(4):349–55.

Boeker KHW, et al. Diagnostic potential of circulating TIMP-1 and MMP-2 as markers of liver fibrosis in patients with chronic hepatitis C. Clin Chim Acta. 2002;316(1–2):71–81.

Bugianesi E, McCullough AJ, Marchesini G. Insulin resistance: a metabolic pathway to chronic liver disease. Hepatology. 2005;42(5):987–1000.

Chevallier M, et al. A histological semiquantitative scoring system for evaluation of hepatic fibrosis in needle liver biopsy specimens: comparison with morphometric studies. Hepatology. 1994; 20(2):349–55.

Cholongitas E, et al. A systematic review of the quality of liver biopsy specimens. Am J Clin Pathol. 2006;125(5):710–21.

Cohen JA, Kaplan MM. The SGOT/SGPT ratio – an indicator of alcoholic liver disease. Dig Dis Sci. 1979;24(11):835–8.

Colloredo G, et al. Impact of liver biopsy size on histological evaluation of chronic viral hepatitis: the smaller the sample, the milder the disease. J Hepatol. 2003;39(2):239–44.

Desmet VJ, et al. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology. 1994;19(6):1513–20.

Dhawan A, Cheeseman P, Mieli-Vergani G. Approaches to acute liver failure in children. Pediatr Transplant. 2004;8(6):584–8.

Donnelly KL, et al. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J Clin Invest. 2005;115(5):1343–51.

Ferraioli G, et al. Accuracy of real-time shear wave elastography for assessing liver fibrosis in chronic hepatitis C: a pilot study. Hepatology. 2012;56(6):2125–33.

Fishbein M, et al. Undetected hepatomegaly in obese children by primary care physicians: a pitfall in the diagnosis of pediatric nonalcoholic fatty liver disease. Clin Pediatr (Phila). 2005;44(2): 135–41.

Fraser CG. Biological variation in clinical chemistry. An update: collated data, 1988–1991. Arch Pathol Lab Med. 1992;116(9):916–23.
Henley KS, Pollard HM. A new method for the determination of glutamic oxalacetic and glutamic pyruvic transaminase in plasma. J Lab Clin Med. 1955;46(5):785–9.
Ip BC, Wang X-D. Non-alcoholic steatohepatitis and hepatocellular carcinoma: implications for lycopene intervention. Nutrients. 2013;6(1):124–62.
Iredale JP, et al. Tissue inhibitor of metalloproteinase-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis. Hepatology. 1996;24(1):176–84.
Ishak K, et al. Histological grading and staging of chronic hepatitis. J Hepatol. 1995;22(6):696–9.
Karlsen TH, Vesterhus M, Boberg KM. Review article: controversies in the management of primary biliary cirrhosis and primary sclerosing cholangitis. Aliment Pharmacol Ther. 2014;39(3): 282–301.
Kelly DA, McKiernan PJ. Metabolic liver disease in the pediatric patient. Clin Liver Dis. 1998;2(1):1–30.
Kelsey MM, et al. Age-related consequences of childhood obesity. Gerontology. 2014;60:222–8.
Kim WR, et al. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. Hepatology. 2008;47(4):1363–70.
Lewandrowski K, Chen A, Januzzi J. Cardiac markers for myocardial infarction. A brief review. Am J Clin Pathol. 2002;118(Suppl):S93–9.
Lindor KD, et al. ACG clinical guideline: primary sclerosing cholangitis. Am J Gastroenterol. 2015;110(5):646–59. quiz 660.
Maharaj B, et al. Sampling variability and its influence on the diagnostic yield of percutaneous needle biopsy of the liver. Lancet. 1986;1(8480):523–5.
Manns M, et al. All-oral daclatasvir plus asunaprevir for hepatitis C virus genotype 1b: a multinational, phase 3, multicohort study. Lancet. 2014;384:1597–160.
Molleston JP, et al. Obese children with steatohepatitis can develop cirrhosis in childhood. Am J Gastroenterol. 2002;97(9):2460–2.
Molleston JP, et al. Histological abnormalities in children with nonalcoholic fatty liver disease and normal or mildly elevated alanine aminotransferase levels. J Pediatr. 2013;164:707–713.e3.
Murawaki Y, et al. Tissue inhibitor of metalloproteinase-1 in the liver of patients with chronic liver disease. J Hepatol. 1997;26(6):1213–9.
Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD single topic conference. Hepatology. 2003;37(5):1202–19.
Nie QH, et al. Inhibiting effect of antisense oligonucleotides phosphorthioate on gene expression of TIMP-1 in rat liver fibrosis. World J Gastroenterol. 2001;7(3):363–9.
Nobili V, Manco M. Therapeutic strategies for pediatric non-alcoholic fatty liver disease: a challenge for health care providers. World J Gastroenterol. 2007;13(18):2639–41.
O'Shea RS, et al. Alcoholic liver disease. Hepatology. 2010;51(1):307–28.
Pacifico L, et al. Nonalcoholic fatty liver disease and carotid atherosclerosis in children. Pediatr Res. 2008;63(4):423–7.
Pearce SG, Thosani NC, Pan J-J. Noninvasive biomarkers for the diagnosis of steatohepatitis and advanced fibrosis in NAFLD. Biomark Res. 2013;1(1):7.
Poynard T, et al. Prognostic value of total serum bilirubin/gamma-glutamyl transpeptidase ratio in cirrhotic patients. Hepatology. 1984;4(2):324–7.
Poynard T, et al. Prospective analysis of discordant results between biochemical markers and biopsy in patients with chronic hepatitis C. Clin Chem. 2004;50(8):1344–55.
Pratt DS, Kaplan MM. Evaluation of abnormal liver-enzyme results in asymptomatic patients. N Engl J Med. 2000;342(17):1266–71.
Roberts EA, Schilsky ML, American Association for Study of Liver Diseases (AASLD). Diagnosis and treatment of Wilson disease: an update. Hepatology. 2008;47(6):2089–111.
Ruhl CE, Everhart JE. Upper limits of normal for alanine aminotransferase activity in the United States population. Hepatology. 2012;55(2):447–54.

Sanyal AJ, et al. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterology. 2001;120(5):1183–92.
Schuppan D, Kim YO. Evolving therapies for liver fibrosis. J Clin Invest. 2013;123(5):1887–901.
Schwimmer JB, et al. Paediatric gastroenterology evaluation of overweight and obese children referred from primary care for suspected non-alcoholic fatty liver disease. Aliment Pharmacol Ther. 2013;38(10):1267–77.
Siest G, et al. Aspartate aminotransferase and alanine aminotransferase activities in plasma: statistical distributions, individual variations, and reference values. Clin Chem. 1975;21(8): 1077–87.
Sokol RJ. Liver cell injury and fibrosis. J Pediatr Gastroenterol Nutr. 2002;35 Suppl 1:S7–10.
Squires RHJ, et al. Acute liver failure in children: the first 348 patients in the pediatric acute liver failure study group. J Pediatr. 2006;148(5):652–8.
Stetler-Stevenson M, et al. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in reactive and neoplastic lymphoid cells. Blood. 1997;89(5):1708–15.
Tanner MS. Mechanisms of liver injury relevant to pediatric hepatology. Crit Rev Clin Lab Sci. 2002;39(1):1–61.
Wai C-T, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology. 2003;38(2):518–26.
Westheim BH, et al. Evaluation of risk factors for bleeding after liver biopsy in children. J Pediatr Gastroenterol Nutr. 2012;55(1):82–7.
Yoshiji H, et al. Tissue inhibitor of metalloproteinases-1 promotes liver fibrosis development in a transgenic mouse model. Hepatology. 2000;32(6):1248–54.
Zachou K, et al. Review article: autoimmune hepatitis – current management and challenges. Aliment Pharmacol Ther. 2013;38(8):887–913.

Bilirubin as a Biomarker in Liver Disease

13

Nahum Méndez-Sánchez, Libor Vítek, Nancy E. Aguilar-Olivos, and Misael Uribe

Contents

N. Méndez-Sánchez (✉) • N.E. Aguilar-Olivos (✉) • M. Uribe (✉)
Liver Research Unit, Medica Sur Clinic & Foundation, Mexico City, Mexico
e-mail: nmendez@medicasur.org.mx; dra.nancy.aguilar@gmail.com; naedith@hotmail.com; muribe@medicasur.org.mx

L. Vítek (✉)
4th Department of Internal Medicine, and Institute of Medical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University in Prague, Prague, Czech Republic
e-mail: L.Vitek@seznam.cz; Libor.Vitek@vfn.cz

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_25

Abstract

Bilirubin had been studied since the eighteenth century because assessment of serum bilirubin concentration is important in the diagnosis and prognosis of patients with liver disorders. All prognostic scores for liver diseases include bilirubin in their calculations, and some studies have shown that bilirubin is an independent biomarker of mortality risk. We know that a high total bilirubin level is an indicator of disease, but nowadays there is evidence indicating the association of a low level with increased risk of diseases, for example, cancer or cardiovascular disorders. Bilirubin is a potential biomarker because its concentration is associated with mortality and may also be associated with the prevention of disease. This chapter will review the usefulness of bilirubin as a marker in liver diseases and their possible use to assess outcomes and risk of other non-liver diseases.

Keywords

Chronic liver diseases • Acute liver failure • Gilbert syndrome • Cardiovascular diseases • Cancer • Autoimmune diseases • Prognosis • Mortality

List of Abbreviations

ABIC	Age–bilirubin–INR–creatinine
ACLF	Acute-on-chronic liver failure
AH	Alcoholic hepatitis
ALF	Acute liver failure
ALFIHMS	ALF in-hospital mortality score
APACHE II score	Acute Physiology and Chronic Health Evaluation II score
BLVR	Biliverdin reductase
CB	Conjugated bilirubin
CO	Carbon monoxide
CTP score	Child–Turcotte–Pugh score
ECBL	Early change in bilirubin level
GAHS	Glasgow alcoholic hepatitis score
HCl	Hydrochloric acid
HELLP	Hemolysis, elevated liver enzyme levels, and low platelet count
HIV	Human immunodeficiency virus
HMOX	Heme oxygenase
HPLC	High-performance liquid chromatography
INR	International normalized ratio
KCH	King's College Hospital
LT	Liver transplantation
mDF	Maddrey discriminant function
MELD	Model for End-Stage Liver Disease

NHANES	National Health and Nutrition Examination Survey
OATP	Organic anion-transporting protein
TcBm	Transcutaneous bilirubin measurement
UCB	Unconjugated bilirubin
UGT1A1	Bilirubin UDP-glucuronosyltransferase

Key Facts of Bilirubin

- Bilirubin is the result of heme breakdown and about 4 mg/kg of body weight is generated each day.
- An elevated level of bilirubinemia may result from overproduction through excessive breakdown of heme; impaired uptake, conjugation, or excretion of bilirubin at hepatocyte; or damaged or obstructed bile ducts blocking flow of unconjugated and conjugated bilirubin.
- The reference range of total serum bilirubin is 0.2–1.2 mg/dL. A total serum bilirubin level of at least 3.0 mg/dL produces the presence of conjunctival icterus, but the differentiation between conjugated and unconjugated hyperbilirubinemia is not possible.
- Bilirubin is lipid soluble; therefore, it binds to albumin in order to be transported in blood and then be conjugated in the hepatocytes. Conjugated bilirubin is also known as "direct" bilirubin.
- Conjugated bilirubin is secreted into the bile flow to reach the distal ileum and colon.

Key Facts of Elevation of the Serum Bilirubin Level

- The etiology of isolated hyperbilirubinemia is usually different from that of the patient with an associated impaired profile of liver enzyme; the latter suggests either a hepatocellular or cholestatic disease.
- Examples of acute hepatocellular diseases included alcohol-induced hepatitis, drug-induced hepatitis, autoimmune hepatitis, and viral hepatitis. Cirrhosis results from chronic hepatocellular insults.
- Drug-induced cholestasis, alpha-1-antitrypsin deficiency, and viral hepatitis are some examples of cholestatic diseases.
- To evaluate a patient with isolated elevation of serum bilirubin level, we have to determine if it is conjugated or unconjugated.
- A proposed threshold of 15% of total bilirubin is used to determine if it is conjugated or unconjugated.
- When we found $\geq$15% of conjugated (direct) bilirubin, we should suspect from biliary obstruction, or rarer Dubin–Johnson or Rotor syndrome (impaired

canalicular export of conjugated bilirubin). In these cases, plasma bilirubin is usually <120 μmol/L (7 mg/dL), and prognosis is similar to unaffected people.
- In case <15% of conjugated (direct) bilirubin is mandatory to evaluate for hemolysis, if negative, we should investigate about drug administration. Finally, in case of any toxic evidence, the workup should focus in inherited disorders like Gilbert syndrome or Crigler–Najjar syndrome types 1 and 2.
- Gilbert syndrome is a condition affecting the *UGT1A1* gene, with an incident of about 6–12%. In this situation bilirubin conjugation is impaired, but plasma bilirubin is usually 68 μmol/L (4mg/dL) in the absence of fasting. A specific treatment is not required because of its benign prognosis.

Definitions of Words and Terms

Acute decompensation	Refers to the acute development of ascites, hemorrhage, encephalopathy, and/or bacterial infections.
Anionic detergents	Any of a class of synthetic compounds whose anions are alkali salts or ammonium salts.
Bα, α-bilirubin	The major form of bilirubin, comprising the isomer (Z,Z)-bilirubin lXα.
Bδ, δ-bilirubin	Bilirubin fraction in a nonenzymatic covalently bound complex with albumin.
Bf, free bilirubin	The fraction of bilirubin that is not bound to albumin or other solubilizing substances.
Enterohepatic circulation of bilirubin and urobilinoids	Under specific conditions, both bile pigments may undergo enterohepatic or even enterosystemic cycling.
Organ system failure	Failure of the liver, kidney, brain, coagulation, circulation, and/or respiration.
Oxidative stress	Disequilibrium in reactive oxygen species production that is not counteracted by the oxidative stress defense system.
Oxidative stress-mediated diseases	Diseases whose pathogenesis is affected by increased oxidative stress, including cardiovascular and inflammatory conditions and cancer.
UGT1A1, bilirubin UDP-glucuronosyltransferase	The key enzyme that determines the bilirubin concentration in the circulation.
UCB, unconjugated bilirubin	Bilirubin non-covalently bound to albumin that does not react directly in the diazo reaction.

Introduction

In this chapter, we outline the importance of bilirubin metabolism and demonstrate how changes in this product are linked to other key biochemical and molecular processes. We review the evidence that bilirubin metabolism can be disrupted by certain diseases, methods for the quantification of bilirubin concentration, and the beneficial and deleterious effects of variations in bilirubin concentration in people with and without acute or chronic liver disease. We propose that bilirubin is a biomarker that can be used to monitor liver function and the consequences of liver dysfunction.

Bilirubin as a Biomarker in Liver Disease

Bilirubin Metabolism

Unconjugated bilirubin (UCB) is the main product of heme catabolism. Its physicochemical properties were the subject of intensive studies dating back to the eighteenth century. In 1847, Rudolf Virchow identified pigment crystals (named hämatoidin) in extravasates of various injured tissues and linked these to the degradation of a blood pigment (Virchow 1847). After its isolation in a pure form, the pigment was named bilirubin.

UCB derives entirely from the degradation of the protoporphyrin portion of the heme group of proteins such as hemoglobin, myoglobin, and cytochrome P-450 via the successive actions of two enzymes, microsomal heme oxygenase (HMOX) and cytosolic biliverdin reductase A (BLVRA) (Fig. 1). Both enzymes are especially abundant in mononuclear and phagocytic cells, hepatocytes, renal tubules, and intestinal mucosa. In the human adult, UCB production is estimated to be 4.4 ± 0.7 mg/kg per day (Berk et al. 1974).

The initial, rate-limiting step of heme catabolism is mediated by HMOX, a phylogenetically very old enzyme that converts heme to biliverdin with the

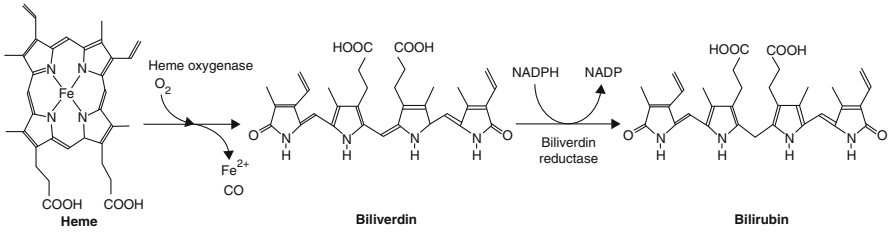

Fig. 1 The heme catabolism pathway. The heme oxidase degrades the heme group producing ferrous iron (Fe^{2+}), carbon monoxide (*CO*), and biliverdin. Biliverdin reductase catalyzes the conversion of biliverdin to bilirubin in the presence of reduced form of nicotinamide adenine dinucleotide phosphate (*NADPH*)

simultaneous release of ferrous iron and carbon monoxide, an important bioactive gas. HMOX1, the inducible isoform of HMOX, is modulated by multiple stimuli that provoke oxidative stress (Ryter et al. 2006).

The reduction of biliverdin to UCB by the cytosolic enzyme BLVRA evolved initially in cyanobacteria but was consolidated in nature with the appearance of placental animals (Schluchter and Glazer 1997). At first glance, this energy-demanding event appears disadvantageous, but it may account for the phylogenic appearance of UCB. The relatively polar biliverdin molecule does not cross the placental barrier to accumulate in the fetus, where it has potentially toxic effects (McDonagh et al. 1981). By contrast, the transplacental passage of nonpolar UCB occurs readily both by diffusion and by energy-driven transporters (Serrano et al. 2002). More importantly, UCB represents a more potent antioxidant than biliverdin in protecting the fetoplacental system from reactive oxygen species. Because the antioxidant action of UCB involves its oxidation to biliverdin, the large excess of BLVRA guarantees rapid regeneration of UCB, thereby allowing low micromolar concentrations of UCB to neutralize millimolar concentrations of toxic oxidant agents (Baranano et al. 2002).

Because of its lipophilic nature, UCB circulates bound to plasma proteins, albumin in particular. At the normal concentration of albumin (40 g/L, 600 μM), UCB can bind up to 30 mg/dL (500 μM) of UCB (Weisiger et al. 2001), leaving the concentration of the unbound pigment (free bilirubin, known as Bf) at $<0.01\%$. This is the only active UCB species that can diffuse across lipid membranes and is responsible for cellular events. Many xenobiotics can compete with UCB for its albumin-binding sites; this has potential clinical consequences such as in newborns treated in the past with sulfonamides (Brodersen 1976). Other transporting molecules, such as α1-fetoprotein, can substitute for albumin in transporting UCB in the circulation (Schieving et al. 2014).

UCB is rapidly transported into the liver cell by facilitated diffusion, which is mediated mainly by the organic anion-transporting (OAT) polypeptides OATP1B1 (Cui et al. 2001) and OATP1B3. Both transporters are involved in the so-called bilirubin "hepatocyte hopping cycle," in which bilirubin conjugates formed in periportal hepatocytes are rerouted into sinusoidal blood via multidrug resistance-associated protein 3 (MRP3), which increases the overall biotransformation capacity. This liver–blood shuttling loop is deficient in Rotor syndrome (van de Steeg et al. 2012).

Inside the liver cell, UCB is solubilized by binding to ligandin (glutathione S-transferase-α or Y protein) (Vander Jagt et al. 1983) or to a fatty acid-binding protein 1. This binding might inhibit glutathione S-transferase-α2 enzymatic activity, which impairs the ability of hepatic cells to detoxify reactive metabolites by glutathione conjugation. Bilirubin and glutathione metabolisms are probably more closely related, as indicated by the higher incidence of reduced glutathione concentration in people with Gilbert syndrome (Boon et al. 2012). Within the intracellular compartment, almost half of the UCB in the hepatocyte is bound to membranes, which provide an enormous sink for UCB (Gollan and Zucker 1996).

Conjugation is normally the rate-limiting step in the overall transfer of UCB from plasma to bile. In humans, the conjugation of UCB occurs mainly with glucuronic acid and is catalyzed by the specific hepatic bilirubin UDP-glucuronosyltransferase (UGT1A1). Congenital underexpression of *UGT1A1* results in mild, chronic, benign fluctuating unconjugated hyperbilirubinemia (Gilbert syndrome). More marked congenital deficiencies of UGT1A1 activity cause a more extreme unconjugated hyperbilirubinemia (Crigler–Najjar syndrome types 1 and 2). Immature UGT1A1 activity is the rule in newborns and contributes substantially to the manifestation of neonatal jaundice (Bosma 2003).

Bilirubin conjugates are secreted into the bile mainly by an active process that is mediated by MRP2, a multispecific organic anion transporter (OAT) in the canalicular membrane (Fouassier et al. 2007). Secreted conjugated bilirubin (CB) reaches the intestine, where anaerobic bacteria cleave bilirubin glucuronosides back to UCB. Some of the UCB may be oxidized to dipyrroles, but most are converted to colorless, water-soluble urobilinoids by bacterial reduction of the conjugated system. Most of the absorbed bile pigments are cleared and re-excreted by the liver, which leads to biliary hypersecretion of UCB (hyperbilirubinbilia) (Vítek and Carey 2003), but about one-third escapes hepatic uptake and appears in the systemic circulation (enterosystemic circulation of UCB) (Gartner et al. 1997). The enterohepatic circulation of UCB can increase when the intestinal microbiome is absent, as in newborns or in patients receiving broad-spectrum antibiotics (Méndez-Sánchez and Uribe 2001; Vitek et al. 2005). Little is known about the precise mechanisms of UCB reduction to urobilinoids and the microbial species involved in this process. Despite the efficiency of this reaction, the nature and structure of bilirubin reductase are still largely unknown. Moreover, the increased reduction of UCB to urobilinoids in the intestinal lumen may have important clinical implications, in particular in newborns lacking intestinal bacteria (Vitek et al. 2000). Although urobilinoid production is highly efficient in adults, only negligible amounts of fecal urobilinoids are present in the intestinal lumen of infants during the first months of life (Vitek et al. 2000).

Methods to Quantify Bilirubin

Assessment of serum bilirubin concentration is important in the diagnosis, treatment, and prognosis of patients with liver disorders. Since bilirubin was isolated for the first time, several methods to measure bilirubin concentration in body fluids have been developed, including spectrophotometry (diazo method), chromatography, and capillary electrophoresis.

Diazo Method

Ehrlich described the diazo reaction in 1883 (Ehrlich 1883). This method is the most frequently used method worldwide for measuring bilirubin concentration. The diazo reaction comprises the addition of diazotized sulfanilic acid to bilirubin, which generates isomeric azodipyrroles that produce a violet color at neutral (Fig. 2).

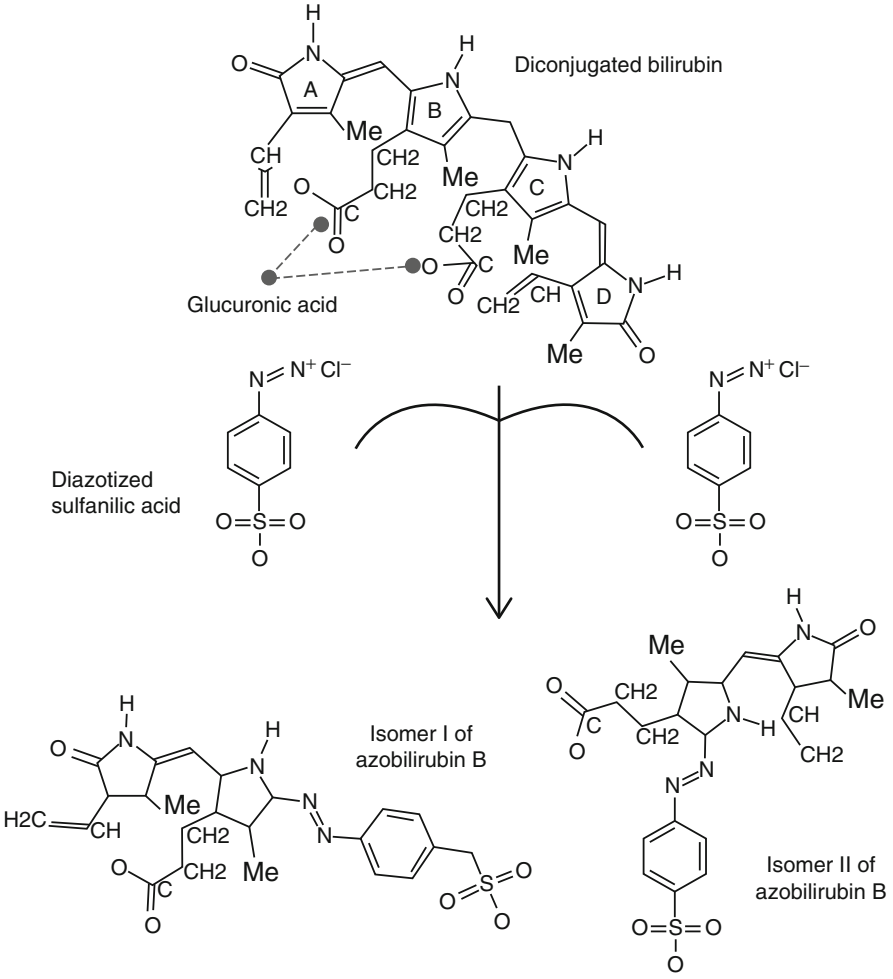

Fig. 2 The diazo reaction of bilirubin glucuronide with diazotized sulfanilic acid to produce isomers I and II of azobilirubin B. The most common method for the determination of bilirubin is the coupling of serum bilirubin with diazotized sulfanilic acid (diazo reagent) to produce an azobilirubin (colored product). Conjugated (direct) and unconjugated (indirect) bilirubin couple with diazo reagent; however, direct bilirubin couples directly with the diazotized reagent, and the indirect portion of bilirubin requires a solubilizing agent. The reaction of conjugated bilirubin with diazo reagent generates isomers I and II of azobilirubin B, while unconjugated bilirubin produces isomers I and II of azobilirubin A

Serum bilirubin concentration is measured using a modification of this method. The conjugated fraction of bilirubin reacts immediately, and this fraction is called "direct" bilirubin. Total bilirubin is measured 30–60 min after the addition of an accelerant (alcohol or caffeine). "Indirect" bilirubin is determined by subtracting the direct fraction from the total bilirubin (Kufer and Scheer 1983).

Nowadays, the reference method for measuring total bilirubin concentration in serum comprises the quantification of the unconjugated, monoconjugated, diconjugated, and delta-bilirubin fractions. Obtaining an exact quantification of direct bilirubin or CB requires the addition of HCl or taurine to maintain the proportion of CB relative to UCB. HCl is added to achieve a pH near 1.0, and taurine forms ditaurobilirubin, which prevents the diazo reaction to UCB (Lott and Doumas 1993). There are several causes of variability and bias in methods to measure direct bilirubin concentration, such as in the calibration, lack of a serum blank, inadequate concentration of HCl, inappropriate use of a bichromatic correction method, and the possible inclusion of wetting agents or surfactants in the reagent (Zieve et al. 1951).

Direct Spectrophotometry

Direct spectrophotometry is used mainly in samples from newborns that have UCB as the predominant fraction. The direct spectrophotometric assay is based on the absorbance of bilirubin at 454 nm, whereas hemoglobin absorbs at both 454 and 528 nm. Bilirubin concentration is obtained by subtracting the absorbance at 528 nm from that at 454 nm. This method eliminates the effect of hemolysis, and the resultant value is attributed primarily to bilirubin. However, some pigments such as carotenoids also absorb at 454 nm, which means that this method is limited to neonates (Kazmierczak et al. 2002).

High-Performance Liquid Chromatography

In 1981, Lauff et al. established a high-performance liquid chromatography (HPLC) method for the separation of least three major bilirubin fractions in human bile (Lauff et al. 1981). Currently, HPLC is considered sensitive and accurate for bilirubin analysis. HPLC methods can be used to separate and quantify the various bilirubin fractions and photoisomers produced during phototherapy. There are several variant HPLC methods for measuring bilirubin concentration. A simple and fast variation uses a Micronex PR-30 column to detect five bilirubin fractions, δ (δ-bilirubin, Bδ), γ (bilirubin diglucuronide), β (bilirubin monoglucuronide), β′ (Z,E- or E,Z-bilirubin lXα), and α (Z,Z-bilirubin lXα), in normobilirubinemic and in conjugated and unconjugated hyperbilirubinemic samples (Adachi et al. 1988). The detection of the Bδ fraction explained the long time required for bilirubin normalization in patients with a very high CB level (Higashijima et al. 1996). HPLC is useful for separating the bilirubin fractions, although this has little clinical value.

Enzymatic Methods

Enzymatic methods use bilirubin oxidase to separate bilirubin fractions in serum, although this method cannot separate all of the bilirubin fractions. The fractions that can be separated enzymatically are CB, UCB, and Bδ. The method involves different conversions to bilirubin fractions catalyzed by bilirubin oxidase at different pH values in the presence or absence of anionic detergents. Thus, in the absence of detergents, UCB and Bδ are oxidized at acidic pH, CB is oxidized at alkaline pH, and UCB is not oxidized at either acidic or alkaline pH. The diazo reaction is coupled

with the enzymatic reaction. Total and CB concentrations are determined using the conventional diazo method, and Bδ concentration is measured using the diazo method after oxidation of the CB fraction. The precision and accuracy of this method are good (Nakayama 1995).

Transcutaneous Measurement of Bilirubin Concentration

Transcutaneous bilirubin measurement (TcBm) is an alternative noninvasive method that provides an instantaneous readout of the cutaneous bilirubin concentration. This technology is based on light absorption by bilirubin as detected by optical spectroscopy. The first TcBm was introduced in 1980 and then several devices have been developed since then. The TcBm is recommended by clinical practice guidelines for the management of hyperbilirubinemia (American Academy of Pediatrics Subcommittee on Hyperbilirubinemia, 2004), and any TcBm device is capable of replacing the invasive blood bilirubin evaluation (Bosschaart et al. 2012). TcBm provides a reasonable estimate of total serum bilirubin concentration in healthy newborns, but it seems to underestimate the total bilirubin concentration, especially when it is $>$10 mg/dL (Taylor et al. 2015). Some benefits of TcBm are the instantaneous information and the painless and noninvasive method (i.e., no need for serum samples).

Bilirubin in Chronic Liver Diseases

Cholestasis is an impairment of bile formation and flow. Cholestasis may be caused by hepatocellular and/or cholangiocellular secretory defects or obstruction of bile ducts by bile duct lesions, stones, or tumors, but may also be related to mixed mechanisms in conditions such as primary biliary cirrhosis/cholangitis (PBC) or primary sclerosing cholangitis (PSC). Acceptable treatment of cholestasis and cholestatic injury requires the identification and targeting of the defective hepatocellular and cholangiocellular secretory mechanisms and/or bile duct lesions.

Cholestatic liver diseases are characterized by impairment of bile flow and accumulation of biliary constituents such as bile acids and bilirubin (Kosters and Karpen 2010). The cholangiopathies are a heterogeneous group of diseases characterized by an inflammatory destruction of bile ducts, which leads to cholestatic liver disease. As mentioned above, the two main cholangiopathies are PBC and PSC. Although PBC is considered to be immune mediated, the causes of both diseases are still unknown. There are several clinically relevant situations in which inflammation either causes or contributes to cholestatic liver diseases. Some of these are evidently linked to autoimmune hepatitis or sepsis, and others are most likely indirect effects of, for example, drugs, posttransplant rejection, total parenteral nutrition (TPN), or systemic conditions such as rheumatoid arthritis (Kosters and Karpen 2010).

An increased concentration of bilirubin glucuronides in blood plasma indicates hepatocellular dysfunction. In septic shock, hyperbilirubinemia is usually a central clinical finding. Conjugated hyperbilirubinemia is the most typical clinical finding in sepsis because it is always included in standard laboratory analyses.

In cases of cholestatic liver injury, after exclusion of the differential diagnoses, the physician must consider drug-induced liver injury (DILI) in patients with an unexplained increase in liver enzyme levels. In such cases, bilirubin concentration is not the main biochemical marker of cholestatic or mixed injury. The clinical presentations of hepatotoxicity that are most readily distinguished are acute hepatocellular injury and cholestatic liver disease. Acute hepatocellular injury is often accompanied by symptoms such as malaise, abdominal pain, and jaundice. Cholestatic liver disease is characterized by jaundice and pruritus, and the alkaline phosphatase level is initially the most prominently elevated of the liver enzymes. Recovery is usually complete but may take several weeks or months (Navarro and Senior 2006).

In healthy people under most conditions, the rate-limiting step for the elimination of bile acids and other cholephiles from the blood is the active transport across the canalicular membrane of the hepatocyte. This is driven by a number of ATP-dependent export pumps (ATP-binding cassette [ABC] transport proteins, also known as ABC transporters). Bile salts are transported by the bile salt export pump (BSEP, ABCB11) (Harada et al. 2003), whereas bilirubin bis-glucuronide, glutathione, divalent bile acid conjugates, and a large variety of other conjugated organic anions are transported by MRP2 (ABCC2) (Chen et al. 2008).

In cholestasis, hepatobiliary transporters are largely responsible for the adaptive coordinated responses in the liver, kidney, and intestine to limit the hepatocellular accumulation of potentially toxic biliary constituents (Geier et al. 2003). As a result of the adaptive transcriptional program in cholestasis, the basolateral bile acid uptake systems are downregulated, and the basolateral export pumps such as MRP3 and MRP4 are induced. Moreover, bile acid hydroxylation and conjugation, which reduce toxicity and increase water solubility for the subsequent alternative elimination via the urine, are stimulated. The central regulator of these adaptive responses is the nuclear receptor for bile acids, FXR (NR1H4), which reduces bile acid uptake into the hepatocyte by downregulating the sodium taurocholate cotransporting polypeptide (Na-dependent bile acid uptake) and OATP1B1 (Na-independent bile acid uptake) (Geier et al. 2003).

Bilirubin in Acute Liver Failure

Acute Liver Failure

Acute liver failure (ALF) is the simultaneous appearance of hepatic encephalopathy and impaired coagulation function (international normalized ratio [INR] $\geq$1.5) in patients with an acute liver insult and in the absence of pre-existing liver disease. The etiologies of ALF are numerous and include acetaminophen toxicity, idiosyncratic drug reactions, viral hepatitis, alcoholic hepatitis, autoimmune hepatitis, Wilson disease, ischemic hepatopathy, Budd–Chiari syndrome, veno-occlusive disease, acute fatty liver of pregnancy/hemolysis, elevated liver enzyme levels, and low platelet count (HELLP) syndrome, toxin exposure, and sepsis (Bernal et al. 2015).

ALF is classified as hyperacute (<7 days), acute (7–21 days), or subacute (>21 days and <26 weeks) (Lidofsky 1993). The liver test profile differs according to the time course. In the hyperacute period, bilirubin levels are low, but the aminotransferase levels are high because this phase is characterized by cell necrosis. The subacute period is characterized by low serum aminotransferase activities and high serum bilirubin levels as a result of a gradual liver injury (Bernal et al. 2015).

A number of prognostic models have been proposed for patients with ALF because of their high mortality, which can be as high as 80% depending on the etiology, and the clinical experience of the reference center in treating patients with ALF without using liver transplantation (LT). The models include the King's College Hospital criteria, Clichy criteria, ALF in-hospital mortality score, serum group-specific component protein levels, liver volume on computed tomography (CT) scanning, blood lactate level, hyperphosphatemia, Acute Physiology and Chronic Health Evaluation II (APACHE) score, serum α-fetoprotein level, the Model for End-Stage Liver Disease (MELD) score, and total bilirubin concentration (Du et al. 2010).

Wlodzimirow et al. published a systematic review of the prognostic indicators of ALF and their ability to predict a poor outcome (Wlodzimirow et al. 2013). The most commonly studied biomarker was total bilirubin concentration (68 studies). The univariate analysis of the association between total bilirubin concentration and mortality found 32 studies with a significant positive association, 35 studies with a significant negative association, and one study with no significant association. Their multivariate analysis found 17 studies with a significant positive association, eight studies with a significant negative association, and one study with no significant association with mortality. The studies that considered different timing of bilirubin measurement or different subgroups of patients showed mixed results: positive, negative, or no association with mortality. A positive association between total bilirubin concentration and mortality was evident in studies that included at least one measurement during the course of the disease or one subgroup of patient.

Alcoholic Hepatitis

Alcoholic hepatitis (AH) is a clinical syndrome comprising liver inflammation, hepatocyte injury, and fibrosis that occurs in the setting of a recent consumption of large amounts of alcohol. The short-term mortality in patients with severe AH is very high: 40–50% (Kim and Kim 2014). Many scoring systems are available to assess the severity and prognosis of AH, including the modified Maddrey discriminant function (mDF), the Child–Turcotte–Pugh (CTP) score, the MELD score, the Glasgow alcoholic hepatitis score (GAHS), the age–bilirubin–INR–creatinine (ABIC) score, the Lille score, and the early change in bilirubin level (ECBL) (Table 1). The purposes of these scoring systems are to estimate the likelihood of short-term survival and to determine whether the patient should be treated with corticosteroids (Lopez-Velazquez et al. 2013).

Table 1 Variables evaluated in the different prognostic scores for alcoholic hepatitis

Score	Bilirubin	ΔBilirubin	Albumin	Creatinine	Urea	PT/INR	Leucocytes	Age
mDF	+	–	–	+	–	+	–	–
CTP	+	–	+	–	–	+	–	–
MELD	+	–	–	+	–	+	–	–
GAHS	+	–	–	–	+	+	+	+
ABIC	+	–	–	+	–	+	–	+
Lille[a]	+	+	+	+	–	+	–	+
ECBL[a]	+	+	–	–	–	–	–	–

mDF Maddrey discriminant function, *CTP* Child–Turcotte–Pugh, *MELD* Model for End-Stage Liver Disease, *GAHS* Glasgow alcoholic hepatitis score, *ABIC* age–bilirubin–INR–creatinine, *ECBL* early change in bilirubin level

[a]Indicators of response to corticosteroid treatment

First described in 1978 and modified in 1989, the mDF score was the first disease-specific score for AH. Patients with an mDF $\geq$32 have a 1-month mortality rate of 35–45% in the absence of specific pharmacotherapy (Ramond et al. 1992).

The CTP score is used for cirrhotic patients. Stages A, B, and C predict mortality rates of 10–15%, 25–30%, and 70–80% at 1 year, respectively. The CTP score may be useful for predicting mortality at 3–6 months; however, because this score is not disease specific, it is not usually used for assessing the severity of AH (Said et al. 2004).

The MELD score is widely used for predicting mortality from end-stage liver disease and for organ allocation in transplant candidates, but it has also been proposed for the assessment of AH (Goyal et al. 2014).

The GAHS was also developed as a disease-specific score. In the first description, it seemed superior to the mDF. Published data indicate that a GAHS $\geq$9 may indicate that the patient would benefit from treatment with corticosteroids (Forrest et al. 2007).

The ABIC score was developed for risk stratification of the risk of death in patients with AH at 90 days and 1 year. The cutoff values of 6.71 and 9.0 are used to separate patients with a low, intermediate, and high risk of death at 90 days (100%, 70%, and 25% survival rate, respectively). The same cutoff values are used to evaluate the risk of death at 1 year (Dominguez et al. 2008).

The Lille model was constructed to identify patients at high risk of death based on the stratification of the risks associated with the use of steroids for AH for 7 days and to predict which patients will not improve and should be considered for other management strategies. Patients with a score >0.45 have a 6-month mortality of about 75% (Louvet et al. 2007).

An ECBL was suggested as a prognostic factor because a decrease in serum bilirubin concentration at 1 week is associated with survival. The original study reported that 73% of patients showed an ECBL and that 83% of them survived (Mathurin et al. 2003).

It is noteworthy that all prognostic models for AH consider the total bilirubin level (Table 1). A comparative study found that the models are best for identifying patients at low risk of death (Papastergiou et al. 2014).

Acute-on-Chronic Liver Disease

Acute-on-chronic liver failure (ACLF) is described as acute decompensated cirrhosis leading to organ/system failure. ACLF has an extremely poor survival, with a 28-day mortality rate of 30–40% (Arroyo et al. 2015).

There are four different diagnostic criteria for ACLF developed by the following groups:

- Asia Pacific Association for the Study of the Liver
- Chinese Medical Association
- European Association for the Study of the Liver and American Association for the Study of Liver Diseases consensus definition
- EASL-Chronic Liver Failure Consortium based on CANONIC study

A comparison of the current diagnostic criteria for ACLF concluded that the different diagnostic criteria produced different patient prognoses. It has been suggested that in ACLF, bilirubin probably passes freely into the brain, where it causes neurotoxic effects, increases hepatic encephalopathy, and could precipitate death (Lopez-Velazquez et al. 2013). Multivariate analyses showed that the levels of CB, UCB, and total bilirubin are independently associated with 7-day mortality ($p = 0.01$, $p = 0.01$, and $p = 0.009$, respectively) and that these levels were significantly more accurate compared with the MELD and Child–Pugh scores. Importantly, the area under the curve obtained from the CB level was significantly higher than those obtained from the UCB and total bilirubin levels (0.751, 0.746, and 0.724, respectively) (all $p < 0.05$) (Fig. 3). The authors demonstrated that a high bilirubin level ($\geq$3.45 mg/dL) at hospital admission predicts short-term mortality in patients with ACLF.

An evaluation of biomarkers also showed that the total bilirubin concentration has a prognostic value (Du et al. 2010); i.e., the risk of an unfavorable evolution of the disease increases with the maximum rising rate of total bilirubin concentration (HR 1.34; 95% CI, 1.161–1.553, $p < 0.001$). This finding could be explained by the relationship between intense jaundice and decreased hepatic detoxification function. In the other studies, which were conducted in vitro, a high level of UCB was neurotoxic and appeared to activate the apoptotic and necrotic pathways in astrocytes and neurons (Brites 2012). Patients with ACLF also present with a low plasma albumin level, which limits the binding of free bilirubin to albumin in plasma and contributes to the increased permeability of the blood–brain barrier because of a high ammonia level (Adachi et al. 1988).

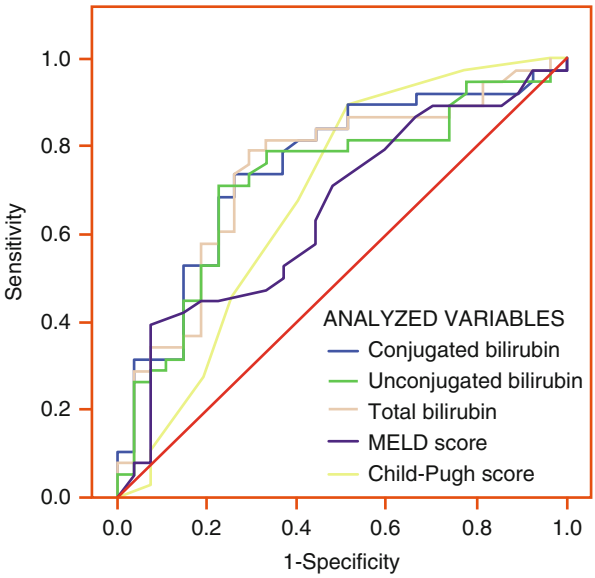

Fig. 3 ROC curve for predicting 1-week mortality in ACLF patients. Conjugated and total bilirubin levels were significantly the best short-term mortality predictors. The AUC was 0.751, 0.746, 0.724, 0.684, and 0.653 for conjugated bilirubin, total bilirubin, unconjugated bilirubin, Child–Pugh score, and MELD score, respectively. (Figure reproduced with permission from Lopez-Velazquez et al. 2013)

Potential Applications to Prognosis, Other Diseases, or Conditions

For a long time, bilirubin has been considered an ominous sign of liver disease. However, a growing body of evidence demonstrates that even in liver diseases, bilirubin exerts protective biological functions, and the same is also true for other systems and organs. In fact, a mildly elevated bilirubin concentration, such as in people with Gilbert syndrome, is associated with protection from cardiovascular, autoimmune, and neuropsychiatric diseases and from cancer (Table 2). On the other hand, extremely high systemic bilirubin concentrations may lead to serious neurological damage, even in adults. In contrast to the currently recognized role of bilirubin as a biomarker, it should be emphasized that a low bilirubin concentration (i.e., <7 μmol/L) should be examined carefully because it is associated with an increased risk of various pathologies (Table 2) and thus should be considered an important risk factor.

Current Lack of Inclusion of Bilirubin as a Marker of Liver Diseases in the Differential Diagnosis

UGT1A1 is the major enzyme that biotransforms UCB into more polar conjugates, and *UGT1A1* is the major gene responsible for systemic bilirubinemia (Lin et al. 2010). Besides endogenous substrates such as UCB, UGT1A1 is also used

Table 2 Bilirubin as a biomarker in various diseases

Organ/system/ disease type	Disease	Ref
Liver	Drug-induced selective impairment of bilirubin metabolism	Lankisch et al. 2006 Lankisch et al. 2009 Palomaki et al. 2009 Sane et al. 2014 Chang et al. 2013
	Sepsis-induced cholestasis	Moseley. 2004
Blood	Hemolytic anemias/ineffective erythropoiesis	Dhaliwal et al. 2004
Heart and vessels	Coronary heart disease	Vitek et al. 2002 Novotny and Vitek. 2003 Vitek and Schwertner. 2007 Schwertner and Vitek. 2008; Lin et al. 2010 Vitek and Ostrow. 2009
	Peripheral atherosclerosis	Novotny and Vitek. 2003 Vitek et al. 2006 Vitek and Schwertner. 2007 Schwertner and Vitek. 2008; Lin et al. 2010 Vitek and Ostrow. 2009
Cancer	Colon cancer	Zucker et al. 2004 Jirásková et al. 2012
	Lung cancer	Lim et al. 2014 Horsfall et al. 2011
	Breast cancer	Wagner et al. 2015
	Ovary cancer	Wagner et al. 2015
Brain	Alzheimer's disease	Kim et al. 2006
	Parkinson's disease	Hatano et al. 2016
	Silent cerebral infarctions	Li et al. 2014
	Leukoaraiosis	Park et al. 2012
	Multiple sclerosis	Peng et al. 2011
	Optic neuritis/neuromyelitis optica	Deng et al. 2013
	Myasthenia gravis	Fuhua et al. 2012
	Amyotrophic lateral sclerosis	Iłzecka and Stelmasiak 2003
	Schizophrenia	Vítek et al. 2010b
	Seasonal depressions	Oren et al. 2002
Immune system/ autoimmunity	Systemic lupus erythematosus	Vítek et al. 2010a
	Rheumatoid arthritis	Fischman et al. 2010
	Psoriasis	Balta et al. 2014
	Crohn's disease	Leníček M et al. 2014
	Ulcerative colitis	Papatheodoridis et al. 1998
	Graft vs. host disease	Lee et al. 2014
	Asthma	Misso et al. 2005
	Experimental autoimmune encephalomyelitis	Liu et al. 2008

to biotransform many xenobiotics, including relatively common medical drugs. Thus, administration of these xenobiotics as typified by atazanavir, the protease inhibitor used in HIV-infected patients, is often associated with mild hyperbilirubinemia (Lankisch et al. 2006), which may become severe in UGT1A1*28 homozygotes (genotypic Gilbert syndrome) (Lankisch et al. 2006). The same bilirubin-increasing effects have been described for several other protease inhibitors, such as indinavir (Lankisch et al. 2009), the topoisomerase I inhibitor irinotecan (Palomaki et al. 2009), or other antivirals such as faldaprevir (Sane et al. 2014). We also note that many drugs can specifically and selectively inhibit both the basolateral and canalicular transport of bilirubin (Chang et al. 2013), and thus, the pharmacological history should always be considered in the differential diagnosis of clinical hyperbilirubinemia. In addition to xenobiotics, bacterial lipopolysaccharide can inhibit the canalicular transport of bilirubin conjugates, and this is believed to be a reason for sepsis-induced cholestasis (Moseley 2004). It should be emphasized that an increase in serum bilirubin concentration occurs very early and often precedes manifestations of other typical signs of sepsis (Moseley 2004).

On the other hand, hyperbilirubinemia occurring with cholestasis that is accompanied by accumulation of bilirubin in the liver tissue is not an ominous sign, but is likely to protect hepatocytes from the deleterious effects of bile acids accumulating within the hepatocytes during cholestasis (Zelenka et al. 2012).

Conditions Associated with Bilirubin Overproduction

Because the major source of bilirubin is hemoglobin from senescent red blood cells, it is not surprising that unconjugated hyperbilirubinemia is associated with bilirubin overproduction relating to hemolysis or inefficient erythropoiesis (Dhaliwal et al. 2004). In chronic hemolytic anemia, systemic bilirubin concentration never exceeds 100 μmol/L, and if a patient manifests with a higher bilirubin level, other causes should be investigated.

Both a high concentration and a lower systemic level of bilirubin should be considered when assessing an individual's risk of oxidative stress-mediated diseases.

Bilirubin and Cardiovascular Diseases

This is especially true for the relationship between bilirubin concentration and cardiovascular diseases, for each micromolar decrease in serum bilirubin concentration significantly increases the risk of cardiovascular disease (Novotny and Vitek 2003), and a bilirubin concentration of 10 μmol/L is the discriminating cutoff (for comprehensive reviews, see Vitek and Schwertner 2007; Schwertner and Vitek 2008; Lin et al. 2010; Vitek and Ostrow 2009). Compared with a bilirubin concentration >10 μmol/L, a serum bilirubin concentration <7 μmol/L increases the risk of

cardiovascular diseases by 30%; the increase in risk is similar to that of high-density lipoprotein cholesterol (Schwertner and Fischer 2000). This is reflected in a new proposed cardiovascular risk calculation algorithm that includes bilirubin concentration as an important modifier (Schwertner and Fischer 2000). It is not surprising that people with phenotypic Gilbert syndrome are protected from the development of ischemic heart disease (Vitek et al. 2002) and peripheral atherosclerosis (Vitek et al. 2006).

Bilirubin and Cancer

Bilirubin is also associated with cancer, although the relationship is slightly more complicated. UGT1A1 is probably responsible for the biotransformation of certain carcinogens; in conditions associated with a decreased UGT1A1 level, such as in Gilbert syndrome, elimination of bilirubin might be impaired. This may explain the contradictory results of certain studies of the relationship between bilirubin level and breast and ovary cancer (for a review, see Wagner et al. 2015). Nevertheless, most of the available data are convincing. The robust data from the large NHANES show that the risk of colon cancer seems to be 75% lower in people with Gilbert syndrome compared with the normobilirubinemic population (Zucker et al. 2004). This was confirmed in a recent study of patients with colorectal cancer, in which UGT1A1*28 allele carrier status was associated with a 20% reduction in colon cancer risk (Jiraskova et al. 2012). A similar negative association between serum bilirubin level and lung cancer risk was reported in recent large British (Horsfall et al. 2011) and Korean (Lim et al. 2014) studies.

Bilirubin and Neuropsychiatric Diseases

Although extreme hyperbilirubinemia is potentially neurotoxic in both newborns (Ostrow et al. 2003) and adults (Chalasani et al. 1997), a low bilirubin level is associated with neurodegenerative diseases. This is certainly true for a number of neurodegenerative/neuropsychiatric diseases, such as Alzheimer's disease (Kim et al. 2006); Parkinson's disease (Hatano et al. 2016), in which bilirubin concentration tends to increase with L-DOPA treatment, which may indicate an equalizing of oxidative stress defenses; silent cerebral infarction (Li et al. 2014); leukoaraiosis (cerebral white matter hyperintensity caused by chronic ischemia of cerebral arterioles) (Park et al. 2012); multiple sclerosis (Peng et al. 2011); optic neuritis/neuromyelitis optica (Deng et al. 2013); myasthenia gravis (Fuhua et al. 2012); amyotrophic lateral sclerosis (Ilzecka and Stelmasiak 2003); and even schizophrenia (Vitek et al. 2010b). Interestingly, a negative association of serum bilirubin concentration with seasonal depression has also been reported (Oren et al. 2002).

Bilirubin and Autoimmune Diseases

Bilirubin has potent immunosuppressive activities and inhibits almost all components of the immune system, including the complement system (Basiglio et al. 2009) and all immune-reactive cells (Jangi et al. 2013). Interestingly, neonates who develop more severe jaundice have difficulty producing antibodies in response to routine vaccination (Nejedla 1970), and patients with severe asthma have a lower bilirubin concentration (Misso et al. 2005). Bilirubin is a potent ligand of the aryl hydrocarbon receptor (Phelan et al. 1998), which contributes to the differentiation of regulatory T cells (Busbee et al. 2013). It is thus not surprising that bilirubin suppressed the course of experimental autoimmune encephalomyelitis (Liu et al. 2008), a model of multiple sclerosis (see also above). These data are also supported by clinical evidence demonstrating a negative relationship similar to those described above for other oxidative stress-mediated diseases between systemic bilirubin concentration and autoimmune diseases. This is especially true for systemic lupus erythematosus (Vitek et al. 2010a), rheumatoid arthritis (Fischman et al. 2010), psoriasis (Balta et al. 2014), Crohn's disease (Lenicek et al. 2014), and probably ulcerative colitis (Papatheodoridis et al. 1998). Additionally, serum bilirubin level, as well as the Gilbert syndrome genotype, has an important effect on graft outcomes in renal transplant patients (Lee et al. 2014). This finding underscores the importance of bilirubin as an immunomodulatory and prognostic factor in autoimmune and neurodegenerative diseases, in which immune components are often intimately involved.

Potential Applications to Prognosis

Bilirubin has traditionally been a marker of liver disease or a hemolytic state. A relationship between bilirubin and other diseases has been shown in recent years, and bilirubin can now be viewed as a potential biomarker.

Bilirubin plays a central role in both acute and chronic liver diseases. All instruments to assess the specific and non-specific prognostic indicators of liver diseases now include bilirubin in their calculations. Some studies that included multivariate analysis have shown that bilirubin is an independent biomarker of mortality risk. Moreover, it has also been shown that bilirubin as an individual parameter also has an independent association with mortality in the short term.

A high total bilirubin level is an indicator of disease, although a low level also indicates an increased risk for disease. Low bilirubin level has been associated with increased risk of cardiovascular disease, and patients with Gilbert disease appear to be protected from this risk. In agreement, some studies have indicated that UCB is associated with a reduction in the prevalence of cardiovascular, autoimmune, and neuropsychiatric diseases and of cancer.

Bilirubin may have a special purpose as a biomarker because its concentration is associated with mortality and may also be associated with the prevention of disease.

This feature looks promising and should stimulate further studies of the role of bilirubin (total and unconjugated) in diseases such as cancer. It is possible to reexplore the role of total bilirubin and its metabolites and to evaluate whether UCB or other fractions can serve as potential biomarkers.

Summary Points

- The bilirubin concentration in blood depends on its production, transformation in the liver tissue, and secretion into the biliary system.
- The diazo method is the most often used technique for bilirubin measurement.
- Bilirubin concentration increases in chronic and acute liver diseases.
- Bilirubin showed a significant positive association with mortality.
- All prognostic models for liver disease take into account the total bilirubin level.
- Both high and low serum/plasma bilirubin concentrations should be considered as biological marker in other non-liver diseases.

Acknowledgements This work was supported by the Medica Sur Clinic and Foundation (NMS) and by the following grants: PRVOUK 4102280002 from the Czech Ministry of Education and RVO VFN64165 from the Czech Ministry of Health (LV).

References

Adachi Y, Inufusa H, Yamashita M, et al. Clinical application of serum bilirubin fractionation by simplified liquid chromatography. Clin Chem. 1988;34:385–8.

American Academy of Pediatrics Subcommittee on Hyperbilirubinemia. Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. Pediatrics. 2004;114:297–316.

Arroyo V, Moreau R, Jalan R, et al. Acute-on-chronic liver failure: a new syndrome that will re-classify cirrhosis. J Hepatol. 2015;62:S131–43.

Balta S, Balta I, Mikhailidis DP, et al. Bilirubin levels and their association with carotid intima media thickness and high-sensitivity C-reactive protein in patients with psoriasis vulgaris. Am J Clin Dermatol. 2014;15:137–42.

Baranano DE, Rao M, Ferris CD, et al. Biliverdin reductase: a major physiologic cytoprotectant. Proc Natl Acad Sci U S A. 2002;99:16093–8.

Basiglio CL, Arriaga SM, Pelusa F, et al. Complement activation and disease: protective effects of hyperbilirubinaemia. Clin Sci (Lond). 2009;118:99–113.

Berk PD, Rodkey FL, Blaschke TF, et al. Comparison of plasma bilirubin turnover and carbon monoxide production in man. J Lab Clin Med. 1974;83:29–37.

Bernal W, Lee WM, Wendon J, et al. Acute liver failure: a curable disease by 2024? J Hepatol. 2015;62:S112–20.

Boon AC, Hawkins CL, Bisht K, et al. Reduced circulating oxidized LDL is associated with hypocholesterolemia and enhanced thiol status in Gilbert syndrome. Free Radic Biol Med. 2012;52:2120–7.

Bosma PJ. Inherited disorders of bilirubin metabolism. J Hepatol. 2003;38:107–17.

Bosschaart N, Kok JH, Newsum AM, et al. Limitations and opportunities of transcutaneous bilirubin measurements. Pediatrics. 2012;129:689–94.

Brites D. The evolving landscape of neurotoxicity by unconjugated bilirubin: role of glial cells and inflammation. Front Pharmacol. 2012;3:88.
Brodersen R. Competitive binding of bilirubin and other substances to plasma albumin: equilibrium studies in vitro. Birth Defects Orig Artic Ser. 1976;12:179–83.
Busbee PB, Rouse M, Nagarkatti M, et al. Use of natural AhR ligands as potential therapeutic modalities against inflammatory disorders. Nutr Rev. 2013;71:353–69.
Chalasani N, Chowdhury NR, Chowdhury JR, et al. Kernicterus in an adult who is heterozygous for Crigler-Najjar syndrome and homozygous for Gilbert-type genetic defect. Gastroenterology. 1997;112:2099–103.
Chang JH, Plise E, Cheong J, et al. Evaluating the in vitro inhibition of UGT1A1, OATP1B1, OATP1B3, MRP2, and BSEP in predicting drug-induced hyperbilirubinemia. Mol Pharm. 2013;10:3067–75.
Chen XM, O'hara SP, Larusso NF. The immunobiology of cholangiocytes. Immunol Cell Biol. 2008;86:497–505.
Cui Y, Konig J, Leier I, et al. Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6. J Biol Chem. 2001;276:9626–30.
Deng J, Liang XM, Zhang XL, et al. Relationship between serum bilirubin levels and optic neuritis. Chin Med J (Engl). 2013;126:3307–10.
Dhaliwal G, Cornett PA, Tierney LM Jr. Hemolytic anemia. Am Fam Physician. 2004;69:2599–606.
Dominguez M, Rincon D, Abraldes JG, et al. A new scoring system for prognostic stratification of patients with alcoholic. Am J Gastroenterol. 2008;103:2747–56.
Du WB, Pan XP, Li LJ. Prognostic models for acute liver failure. Hepatobiliary Pancreat Dis Int. 2010;9:122–8.
Ehrlich P. Sulfadiazobensol, ein Reagens auf Bilirubin. Centr Klin Med. 1883;4:721–3.
Fischman D, Valluri A, Gorrepati VS, et al. Bilirubin as a protective factor for rheumatoid arthritis: an NHANES study of 2003–2006 data. J Clin Med Res. 2010;2:256–60.
Forrest EH, Morris AJ, Stewart S, et al. The glasgow alcoholic hepatitis score identifies patients who may benefit from corticosteroids. Gut. 2007;56:1743–6.
Fouassier L, Beaussier M, Schiffer E, et al. Hypoxia-induced changes in the expression of rat hepatobiliary transporter genes. Am J Physiol Gastrointest Liver Physiol. 2007;293:G25–35.
Fuhua P, Xuhui D, Zhiyang Z, et al. Antioxidant status of bilirubin and uric acid in patients with myasthenia gravis. Neuroimmunomodulation. 2012;19:43–9.
Gärtner U, Goeser T, Wolkoff AW. Effect of fasting on the uptake of bilirubin and sulfobromophthalein by the isolated perfused rat liver. Gastroenterology. 1997;113:1707–13.
Geier A, Dietrich CG, Voigt S, et al. Effects of proinflammatory cytokines on rat organic anion transporters during toxic liver injury and cholestasis. Hepatology. 2003;38:345–54.
Gollan JL, Zucker SD. A new voyage of discovery: transport through the hepatocyte. Trans Am Clin Climatol Assoc. 1996;107:48–55.
Goyal SK, Dixit VK, Jain AK, et al. Assessment of the Model for End-stage Liver Disease (MELD) score in predicting prognosis of patients with alcoholic hepatitis. J Clin Exp Hepatol. 2014;4:19–24.
Harada K, Ohira S, Isse K, et al. Lipopolysaccharide activates nuclear factor-kappaB through toll-like receptors and related molecules in cultured biliary epithelial cells. Lab Invest. 2003;83:1657–67.
Hatano T, Saiki S, Okuzumi A, et al. Identification of novel biomarkers for Parkinson's disease by metabolomic technologies. J Neurol Neurosurg Psychiatry. 2016;87:295–301.
Higashijima H, Yamashita H, Makino I, et al. Significance of serum delta bilirubin during obstructive jaundice in dogs. J Surg Res. 1996;66:119–24.
Horsfall LJ, Rait G, Walters K, et al. Serum bilirubin and risk of respiratory disease and death. JAMA. 2011;305:691–7.
Iłzecka J, Stelmasiak Z. Serum bilirubin concentration in patients with amyotrophic lateral sclerosis. Clin Neurol Neurosurg. 2003;105:237–40.

Jangi S, Otterbein L, Robson S. The molecular basis for the immunomodulatory activities of unconjugated bilirubin. Int J Biochem Cell Biol. 2013;45:2843–51.
Jirásková A, Novotný J, Novotný L, et al. Association of serum bilirubin and promoter variations in HMOX1 and UGT1A1 genes with sporadic colorectal cancer. Int J Cancer. 2012;131: 1549–55.
Kazmierczak SC, Robertson AF, Catrou PG, et al. Direct spectrophotometric method for measurement of bilirubin in newborns: comparison with HPLC and an automated diazo method. Clin Chem. 2002;48:1096–7.
Kim W, Kim DJ. Severe alcoholic hepatitis-current concepts, diagnosis and treatment options. World J Hepatol. 2014;6:688–95.
Kim TS, Pae CU, Yoon SJ, et al. Decreased plasma antioxidants in patients with Alzheimer's disease. Int J Geriatr Psychiatry. 2006;21:344–8.
Kosters A, Karpen SJ. The role of inflammation in cholestasis: clinical and basic aspects. Semin Liver Dis. 2010;30:186–94.
Kufer W, Scheer H. The diazo reaction of bilirubin: structure of the yellow products: studies on plant bile pigments-14. Tetrahedron. 1983;39:1887–92.
Lankisch TO, Moebius U, Wehmeier M, et al. Gilbert's disease and atazanavir: from phenotype to UDP-glucuronosyltransferase haplotype. Hepatology. 2006;44:1324–32.
Lankisch TO, Behrens G, Ehmer U, et al. Gilbert's syndrome and hyperbilirubinemia in protease inhibitor therapy – an extended haplotype of genetic variants increases risk in indinavir treatment. J Hepatol. 2009;50:1010–8.
Lauff JJ, Kasper ME, Ambrose RT. Separation of bilirubin species in serum and bile by high-performance reversed-phase liquid chromatography. J Chromatogr. 1981;226:391–402.
Lee JP, do Kim H, Yang SH, et al. Serum bilirubin affects graft outcomes through UDP-glucuronosyltransferase sequence variation in kidney transplantation. PLoS One. 2014;9:e93633.
Leníček M, Duricová D, Hradsky O, et al. The relationship between serum bilirubin and Crohn's disease. Inflamm Bowel Dis. 2014;20:481–7.
Li RY, Cao ZG, Zhang JR, et al. Decreased serum bilirubin is associated with silent cerebral infarction. Arterioscler Thromb Vasc Biol. 2014;34:946–51.
Lidofsky SD. Liver transplantation for fulminant hepatic failure. Gastroenterol Clin North Am. 1993;22:257–69.
Lim JE, Kimm H, Jee SH. Combined effects of smoking and bilirubin levels on the risk of lung cancer in Korea: the severance cohort study. PLoS One. 2014;9, e103972.
Lin JP, Vitek L, Schwertner HA. Serum bilirubin and genes controlling bilirubin concentrations as biomarkers for cardiovascular disease. Clin Chem. 2010;56:1535–43.
Liu Y, Li P, Lu J, et al. Bilirubin possesses powerful immunomodulatory activity and suppresses experimental autoimmune encephalomyelitis. J Immunol. 2008;181:1887–97.
López-Velázquez JA, Chávez-Tapia NC, Ponciano-Rodríguez G, et al. Bilirubin alone as a biomarker for short-term mortality in acute-on-chronic liver failure: an important prognostic indicator. Ann Hepatol. 2013;13:98–104.
Lott JA, Doumas BT. "Direct" and total bilirubin tests: contemporary problems. Clin Chem. 1993;39:641–7.
Louvet A, Naveau S, Abdelnour M, et al. The Lille model: a new tool for therapeutic strategy in patients with severe alcoholic hepatitis treated with steroids. Hepatology. 2007;45:1348–54.
Mathurin P, Abdelnour M, Ramond MJ, et al. Early change in bilirubin levels is an important prognostic factor in severe alcoholic hepatitis treated with prednisolone. Hepatology. 2003;38:1363–9.
McDonagh AF, Palma LA, Schmid R. Reduction of biliverdin and placental transfer of bilirubin and biliverdin in the pregnant guinea pig. Biochem J. 1981;194:273–82.
Méndez-Sánchez N, Uribe M. Intestinal motility and bacterial overgrowth in patients with gallstones. Gastroenterology. 2001;120:1310–1.

Misso NL, Brooks-Wildhaber J, Ray S, et al. Plasma concentrations of dietary and nondietary antioxidants are low in severe asthma. Eur Respir J. 2005;26:257–64.
Moseley RH. Sepsis and cholestasis. Clin Liver Dis. 2004;8:83–94.
Nakayama K. Differences between enzymatic and diazo methods for measuring direct bilirubin. Eur J Clin Chem Clin Biochem. 1995;33:513–7.
Navarro VJ, Senior JR. Drug-related hepatotoxicity. N Engl J Med. 2006;354:731–9.
Nejedlá Z. The development of immunological factors in infants with hyperbilirubinemia. Pediatrics. 1970;45:102–4.
Novotný L, Vítek L. Inverse relationship between serum bilirubin and atherosclerosis in men: a meta-analysis of published studies. Exp Biol Med (Maywood). 2003;22:568–71.
Oren DA, Desan PH, Boutros N, et al. Effects of light on low nocturnal bilirubin in winter depression: a preliminary report. Biol Psychiatry. 2002;51:422–5.
Ostrow JD, Pascolo L, Shapiro SM, et al. New concepts in bilirubin encephalopathy. Eur J Clin Invest. 2003;33:988–97.
Palomaki GE, Bradley LA, Douglas MP, et al. Can UGT1A1 genotyping reduce morbidity and mortality in patients with metastatic colorectal cancer treated with irinotecan? An evidence-based review. Genet Med. 2009;11:21–34.
Papastergiou V, Tsochatzis EA, Pieri G, et al. Nine scoring models for short-term mortality in alcoholic hepatitis: cross-validation in a biopsy-proven cohort. Aliment Pharmacol Ther. 2014;39:721–32.
Papatheodoridis GV, Hamilton M, Mistry PK, et al. Ulcerative colitis has an aggressive course after orthotopic liver transplantation for primary sclerosing cholangitis. Gut. 1998; 43:639–44.
Park BJ, Shim JY, Lee HR, et al. Association between serum total bilirubin level and leukoaraiosis in Korean adults. Clin Biochem. 2012;45:289–92.
Peng F, Deng X, Yu Y, et al. Serum bilirubin concentrations and multiple sclerosis. J Clin Neurosci. 2011;18:1355–9.
Phelan D, Winter GM, Rogers WJ, et al. Activation of the Ah receptor signal transduction pathway by bilirubin and biliverdin. Arch Biochem Biophys. 1998;357:155–63.
Ramond MJ, Poynard T, Rueff B, et al. A randomized trial of prednisolone in patients with severe alcoholic hepatitis. N Engl J Med. 1992;326:507–12.
Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev. 2006;86:583–650.
Said A, Williams J, Holden J, et al. Model for end stage liver disease score predicts mortality across a broad spectrum of liver disease. J Hepatol. 2004;40:897–903.
Sane RS, Steinmann GG, Huang Q, et al. Mechanisms underlying benign and reversible unconjugated hyperbilirubinemia observed with faldaprevir administration in hepatitis C virus patients. J Pharmacol Exp Ther. 2014;351:403–12.
Schieving JH, de Vries M, van Vugt JM, et al. Alpha-fetoprotein, a fascinating protein and biomarker in neurology. Eur J Paediatr Neurol. 2014;18:243–8.
Schluchter WM, Glazer AN. Characterization of cyanobacterial biliverdin reductase. Conversion of biliverdin to bilirubin is important for normal phycobiliprotein biosynthesis. J Biol Chem. 1997;272:13562–9.
Schwertner HA, Fischer Jr JR. Comparison of various lipid, lipoprotein, and bilirubin combinations as risk factors for predicting coronary artery disease. Atherosclerosis. 2000;150:381–7.
Schwertner HA, Vítek L. Gilbert syndrome, UGT1A1*28 allele, and cardiovascular disease risk: possible protective effects and therapeutic applications of bilirubin. Atherosclerosis. 2008;198:1–11.
Serrano MA, Bayón JE, Pascolo L, et al. Evidence for carrier-mediated transport of unconjugated bilirubin across plasma membrane vesicles from human placental trophoblast. Placenta. 2002;23:527–35.
Taylor JA, Burgos AE, Flaherman V, et al. Discrepancies between transcutaneous and serum bilirubin measurements. Pediatrics. 2015;135:224–31.

van de Steeg E, Stránecký V, Hartmannová H, et al. Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver. J Clin Invest. 2012;122:519–28.

Vander Jagt DL, Dean VL, Wilson SP, et al. Regulation of the glutathione S-transferase activity of bilirubin transport protein (ligandin) from human liver. Enzymic memory involving protein-protein interactions. J Biol Chem. 1983;258:5689–94.

Virchow R. Die pathologischen Pigmente. Arch Pathol Anat Physiol Klin Med. 1847;1:379.

Vítek L, Carey MC. Enterohepatic cycling of bilirubin as a cause of 'black' pigment gallstones in adult life. Eur J Clin Invest. 2003;33:799–810.

Vítek L, Ostrow JD. Bilirubin chemistry and metabolism; harmful and protective aspects. Curr Pharm Des. 2009;15:2869–83.

Vítek L, Schwertner HA. The heme catabolic pathway and its protective effects on oxidative stress-mediated diseases. Adv Clin Chem. 2007;43:1–57.

Vítek L, Kotal P, Jirsa M, et al. Intestinal colonization leading to fecal urobilinoid excretion may play a role in the pathogenesis of neonatal jaundice. J Pediatr Gastroenterol Nutr. 2000;30:294–8.

Vítek L, Jirsa M, Brodanová M, et al. Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels. Atherosclerosis. 2002;160:449–56.

Vítek L, Zelenka J, Zadinová M, et al. The impact of intestinal microflora on serum bilirubin levels. J Hepatol. 2005;42:238–43.

Vítek L, Novotný L, Sperl M, et al. The inverse association of elevated serum bilirubin levels with subclinical carotid atherosclerosis. Cerebrovasc Dis. 2006;21:408–14.

Vítek L, Muchová L, Jančová E, et al. Association of systemic lupus erythematosus with low serum bilirubin levels. Scand J Rheumatol. 2010a;39:480–4.

Vítek L, Novotná M, Lenícek M, et al. Serum bilirubin levels and UGT1A1 promoter variations in patients with schizophrenia. Psychiatry Res. 2010b;178:449–50.

Wagner KH, Wallner M, Mölzer C, et al. Looking to the horizon: the role of bilirubin in the development and prevention of age-related chronic diseases. Clin Sci (Lond). 2015;129:1–25.

Weisiger RA, Ostrow JD, Koehler RK, et al. Affinity of human serum albumin for bilirubin varies with albumin concentration and buffer composition: results of a novel ultrafiltration method. J Biol Chem. 2001;276:29953–60.

Wlodzimirow KA, Eslami S, Abu-Hanna A, et al. A systematic review on prognostic indicators of acute on chronic liver failure and their predictive value for mortality. Liver Int. 2013;33:40–52.

Zelenka J, Muchova L, Zelenkova M, et al. Intracellular accumulation of bilirubin as a defense mechanism against increased oxidative stress. Biochimie. 2012;94:1821–7.

Zucker SD, Horn PS, Sherman KE. Serum bilirubin levels in the U.S. population: gender effect and inverse correlation with colorectal cancer. Hepatology. 2004;40:827–35.

Zieve L, Hill E, Hanson M, et al. Normal and abnormal variations and clinical significance of the one-minute and total serum bilirubin determinations. J Lab Clin Med. 1951;38:446–69.

AST-to-Platelet Ratio Index (APRI) as Marker in Liver Disease

14

Agnieszka Bakula and Maciej Dadalski

Contents

Abstract

Liver fibrosis observed in various chronic disorders is a risk factor of unfavorable prognosis and usually leads to cirrhosis. To date, liver biopsy is the gold standard for assessment of fibrosis. Its invasiveness and risk of hemorrhage incline to looking for noninvasive markers of liver fibrosis. The APRI index includes two simple and cheap laboratory tests performed routinely in clinical practice. Usefulness of this marker seems to depend on the etiology of liver damage. The predictive accuracy of APRI for significant fibrosis and cirrhosis was tested by the areas under the receiver operating characteristic curves (AUROC). The APRI

A. Bakula (✉) • M. Dadalski
Department of Gastroenterology, Hepatology, Nutrition Disorders and Paediatrics, The Children's Memorial Health Institute, Warsaw, Poland
e-mail: agnieszkabakula@gmail.com; a.bakula@czd.pl; m.dadalski@czd.pl

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_2

score correlates significantly to fibrosis stage in patients with chronic hepatitis C. The APRI in patients with autoimmune hepatitis, chronic hepatitis B, and alcoholic liver disease is controversial and does not seem to have a diagnostic value in significant fibrosis. It showed promising results for predicting the presence of fibrosis in pediatric patients with NAFLD. Transient elastography, a noninvasive and objective but expensive method, showed higher performance in diagnosing significant fibrosis than APRI.

Keywords

APRI • Liver fibrosis • Cirrhosis • Viral hepatitis • Liver biopsy

List of Abbreviations

AIH	Autoimmune hepatitis
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
APRI	Aspartate aminotransferase-to-platelet ratio index
AST	Aspartate aminotransferase
ATD	α-1-antitrypsin deficiency
AUROC	Area under the receiver operating characteristic curve
CHB	Chronic hepatitis B
CHC	Chronic hepatitis C
HAART	Highly active antiretroviral therapy
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
INR	International normalized ratio
NAFLD	Nonalcoholic fatty liver disease
NPV	Negative predictive value
OV	Esophageal varices
PBC	Primary biliary cirrhosis
PPV	Positive predictive value
UDCA	Ursodeoxycholic acid

Key Facts

1. Fibrosis is observed in various chronic disorders of the liver.
2. Liver biopsy is regarded as the best method in the diagnostics of liver fibrosis.
3. Liver biopsy is an invasive procedure with a risk of hemorrhage, but the management and prognosis often depend on its result.
4. A noninvasive, painless and cheap test for assessing liver fibrosis is needed.
5. APRI index as a combination of serum parameters, AST and platelet count, is an easily available marker.
6. Its usefulness in diagnosing liver fibrosis of various origin was described.

Definitions of Words and Terms

APRI	The aspartate aminotransferase-to-platelet ratio index is inexpensive, simple marker with value in predicting liver fibrosis of various origin.
Ishak score	The system assessing the histopathological degree of liver fibrosis from 0 to 6 in patients with chronic liver infection.
Liver cirrhosis	A final stage of fibrosis with severe scarring of the liver and poor liver function tests. It is usually caused by alcohol, viruses, autoimmune liver diseases, and toxic metals.
Liver fibrosis	Histological change caused by chronic liver inflammation with increased synthesis of extracellular matrix but without lobular regeneration of the liver.
METAVIR score	A system assessing the degree of inflammation and fibrosis from 0 to 4 in liver biopsy specimen
Transient elastography	Fibroscan is a new noninvasive, painless method that quantifies liver fibrosis. It measures liver elasticity using both ultrasound and low-frequency elastic waves.

Introduction

Liver fibrosis is a result of chronic damage caused by various factors. The major etiologies in adults are chronic infections with hepatitis B (HBV), C (HCV), autoimmune hepatitis, nonalcoholic and alcoholic fatty liver disease, and alpha-1-antitrypsin deficiency. The other metabolic causes such us Wilson disease (WD) or cholesterol ester storage disease (CESD) are uncommon. As many as 25–45% of 400 million patients with chronic HBV infection die of cirrhosis and its complications (Sorrell et al. 2009), while 40% of patients with cirrhosis remain asymptomatic (Fattovich et al. 1997). To date, liver biopsy has been regarded as the gold standard for assessment of fibrosis. It allows to assess staging of liver fibrosis, grade of inflammation, steatosis, iron overload, and copper overload and to obtain information on comorbidities, e.g., autoimmune hepatitis. The treatment decisions in chronic viral hepatitis are usually guided by the results of liver biopsy. However, there are some limitations of this procedure. The percutaneous biopsy is a highly invasive, expensive procedure, prone to sampling errors and underestimation of cirrhosis. Significant hemorrhage after percutaneous liver biopsy may lead to death in exceptional cases. The result of percutaneous biopsy depends on the size of liver biopsy, number of biopsies, and pathomorphologist's experience. It inclines to looking for noninvasive markers identifying patients with liver fibrosis and suitable for serial

assessment during therapy. The aspartate aminotransferase-to-platelet ratio index (APRI) was first reported in 2003 by Wai et al. in a group of patients with liver fibrosis due to chronic hepatitis C (Wai et al. 2003). The APRI as a combination of serum parameters is a cheap, simple, and easily available marker. Both laboratory parameters are performed regularly in clinical practice. AST level usually rises with the progression of liver fibrosis and hepatocytes damage and reduced clearance. The changes of portal blood flow, splenomegaly, and altered production of thrombopoietin lead to a decrease in platelet counts. That is why APRI should correlate with progression of liver disease. There are some limitations of this parameter. The AST level rises late in disease progression. Similarly, platelet counts fall relatively late. On the other hand, AST levels are usually normal in compensated cirrhosis. The limitations of AST depend on the laboratory method of AST assessment (Tables 1, 2, 3, 4, 5, 6, 7, and 8).

Usefulness of this marker seems to be dependent on the etiology of liver damage. The idea that APRI could obviate liver biopsy was analyzed by numerous researchers in many studies concerning various liver disorders, but the results were conflicting. Most authors suggest that the sensitivity of APRI depends on the etiology of liver injury. The predictive accuracy of APRI in significant fibrosis and cirrhosis was tested by the areas under the receiver operating characteristic curves (AUROC).

Diagnostic ability of APRI for prediction of liver fibrosis was assessed in patients with various liver disorders. In chronic hepatitis C (CHC), the sensitivity of APRI for significant fibrosis ranges between 41% and 91% and for cirrhosis between 38.4% and 65.8%. The specificity for fibrosis ranges between 47% and 95% and for cirrhosis between 86.7% and 93%. The accuracy of APRI to detect significant fibrosis and cirrhosis in patients with chronic hepatitis C was assessed as 60–82% and 60–88.4% compared to 76.1% and 79.2% in chronic hepatitis B, respectively.

Table 1 Discriminant ability of APRI in patients with HCV infection

Author	Compared groups		AUROC
Chun-Tao Wai et al.	Non significant fibrosis	Significant fibrosis	0.8
	No cirrhosis	Cirrhosis	0.89
Snyder et al.	Nonsignificant fibrosis	Significant fibrosis	0.889
Loaeza-del-Castillo et al.	Fibrosis stages 0–1	Fibrosis stages 2–4	0.776
	Fibrosis stages 0–2	Fibrosis stages 3–4	0.803
	No cirrhosis	Cirrhosis	0.83
Shaheen and Myers et al.	Non significant fibrosis	Significant fibrosis	0.76
	No cirrhosis	Cirrhosis	0.82
Ngo et al.	Survival without HCV complication	Survival with HCV complication	0.87
	Survival without HCV death	Survival with HCV death	0.73
Yu et al.	No HCC	HCC	0.715–0.87
	Overall survival		0.53–0.87

Table 2 Discriminant ability of APRI in patients with HCV/HIV coinfection

Author	Compared groups		AUROC
Macias et al.	Non significant fibrosis	Significant fibrosis	0.73–0.8
	No cirrhosis	Cirrhosis	0.77–0.8

Table 3 Discriminant ability of APRI in patients with alcoholic liver disease

Author	Compared groups		AUROC
Lieber et al.	No cirrhosis	Cirrhosis	0.79
	Fibrosis stages 0–1	Fibrosis stages 2–4	0.7
Naveau et al.	No cirrhosis	Cirrhosis	0.67
	Fibrosis stages 0–1	Fibrosis stages 2–4	0.59
Nguyen-Khac et al.	Fibrosis stages 0–2	Fibrosis stages 3–4	0.43
	Fibrosis stages 0–1	Fibrosis stages 2–4	0.54
	No fibrosis	Any fibrosis	0.76

Table 4 Discriminant ability of APRI in patients with nonalcoholic fatty liver disease

Author	Compared groups		AUROC
Loaeza-del-Castillo et al.	Fibrosis stages 0–1	Fibrosis stages 2–4	0.564
	Fibrosis stages 0–2	Fibrosis stages 3–4	0.568
	No cirrhosis	Cirrhosis	0.599
Kim et al.	No fibrosis	Fibrosis	0.875

Table 5 Discriminant ability of APRI in patients with autoimmune hepatitis

Author	Compared groups		AUROC
Loaeza-del-Castillo et al.	Fibrosis stages 0–1	Fibrosis stages 2–4	0.602
	Fibrosis stages 0–2	Fibrosis stages 3–4	0.532
	No cirrhosis	Cirrhosis	0.599

Table 6 Discriminant ability of APRI in patients with α-1-antitrypsin deficiency

Author	Compared groups		AUROC
Bakula et al.	Fibrosis stages 0–1	Fibrosis stages 2–4	0.74
	No cirrhosis	Cirrhosis	0.51

Table 7 Discriminant ability of APRI in patients with biliary atresia

Author	Compared groups		AUROC
Yang et al.	Fibrosis stages 0–1	Fibrosis stages 2–4	0.75
	No cirrhosis	Cirrhosis	0.81
	No postoperative jaundice	Postoperative jaundice	0.67

Table 8 Discriminant ability of APRI in patients with HBV infection

Author	Compared groups		AUROC
Jin W et al.	Non significant fibrosis	Significant fibrosis	0.79
	No cirrhosis	Cirrhosis	0.75
Lebensztejn et al.	Fibrosis stages 0–2	Fibrosis stages 3–4	0.748
Celikbilek et al.	Nonsignificant fibrosis	Significant fibrosis	0.62
	No cirrhosis	Cirrhosis	0.67

Potential Applications to Prognosis, Other Diseases, or Conditions

Chronic Hepatitis C

It is estimated that approximately 3% of the world population is infected with HCV. In the United States, it is a leading cause of liver transplantation and liver-related mortality. Most patients are at risk of significant liver fibrosis and cirrhosis.

Wai et al., found that APRI >1.5 predicted significant fibrosis (Ishak score ≥3) and cirrhosis (Ishak score ≥5) with a high degree of accuracy: AUROC of 0.8–0.88 and 0.89–0.94, respectively (Wai et al. 2003). In a cohort study of adult, treatment-naïve patients with CHC, APRI predicted accurately fibrosis and cirrhosis in 51% and 81%, respectively.

Since that report, an increasing number of analyses have assessed usefulness of APRI in the diagnosis of liver fibrosis in HCV-infected patients. Other studies found lower accuracy of APRI.

In a systematic review of 22 studies ($n = 4{,}266$) in 2007, the summary AUCs of APRI for significant fibrosis were 0.76 and for cirrhosis were 0.82 (Shaheen and Myers 2007). An APRI cutoff value of 0.5 had acceptable accuracy to exclude significant fibrosis (81% sensitivity, 50% specificity) and allowed to avoid at least 30% of biopsies. For cirrhosis, the cutoff value of 1.0 had sensitivity of 76% and specificity of 71%. The accuracy of APRI to detect cirrhosis was greater in younger patients, in studies with higher proportion of males and in patients coinfected with HIV/HCV. As a predictor of fibrosis in HCV patients, APRI seemed to have moderate utility but may have played a role in exclusion of significant fibrosis in at least one-third and cirrhosis in three quarters of patients.

In another meta-analysis published by Lin et al in 2011, which evaluated 40 studies ($n = 8{,}739$) comparing APRI with liver biopsy, the summary AUROC of APRI for diagnosis of significant fibrosis and cirrhosis was 0.77 and 0.83, respectively (Lin et al. 2011). The accuracy was less than that described by Wai et al. An APRI threshold of 0.7 for significant fibrosis had sensitivity of 77% and specificity of 72%, and for cirrhosis, the threshold of 1.0 had sensitivity of 76% and specificity of 72%, respectively. A cutoff of 2.0 had better specificity (92%) but was less sensitive (46%). The accuracy of APRI to identify fibrosis compared to liver biopsy in HCV-infected patients was assessed in that meta-analysis as moderate. In contrast

to the previous analysis, APRI was found to be less accurate in HIV/HCV coinfection than in HCV monoinfection.

The usefulness of APRI to predict the risk of hepatocellular carcinoma (HCC) and mortality in HCV-infected patients after interferon-based therapy was also explored. The APRI and other simple noninvasive parameters like platelet count, AST, and alpha-fetoprotein were evaluated 6 months after the end of treatment in the group of 776 chronic hepatitis C patients treated with interferon and at baseline in 562 untreated patients, during follow-up at 4.75 (1.0–12.2) and 5.15 (1.0–16) years, respectively. The APRI was significantly higher in all patients after interferon treatment who developed HCC and in those who died. Based on the ROC analysis, AUC of APRI to predict the long-term outcome was 0.649–0.909. The APRI of >0.75 was correlated with the incidence of posttreatment risk of HCC and mortality for all patients treated with interferon. In the subgroup of patients who did not respond to the therapy, the cumulative incidence of HCC and mortality was significantly higher for APRI >1.5, among those who responded to interferon- for APRI >0.5, and in the subgroup without cirrhosis- for APRI >0.6. To conclude, APRI can predict long-term follow-up results with a high degree of accuracy in patients treated with interferon. In the subgroup of patients with preexisting cirrhosis, the power of APRI for predicting HCC and mortality was not strong enough. The limitation of the study was the change in hepatic fibrosis and APRI after antiviral treatment in the long-term follow-up.

APRI levels are important to reduce the number of liver biopsies as, according to the Mata-Marin results, patients with APRI of less than 0.4 very rarely have significant liver fibrosis (Mata-Marin et al. 2009).

HCV-infected patients were also examined with paired liver biopsies to find out if longitudinal changes in APRI may correlate with an increase in the stage of fibrosis. The APRI was examined together with another noninvasive marker of liver fibrosis, FIB-4. Both parameters predicted two-stage progression of liver fibrosis in the second biopsy. The ΔAPRI of 0.18 may suggest progression in fibrosis of at least one stage with positive predictive value (PPV) of 80% and negative predictive value (NPV) of 67%. The ΔAPRI and ΔFIB-4 could be useful for clinicians to reconsider the decision on antiviral therapy. However, a limitation of the study was a small number of patients and its retrospective nature. Interestingly, in the initial biopsy, APRI did not predict the progression of liver fibrosis.

The AUROC of APRI to detect significant fibrosis and cirrhosis in HCV-infected patients is worse than a fibroscan (transient elastography) (Mummadi et al. 2010). According to a meta-analysis by Shaheen et al., AUROC was 0.83 and 0.95, respectively (Shaheen et al. 2007). But still, fibroscan being expensive had a limited availability, especially in regions with high prevalence of HCV infection.

Patients with liver cirrhosis due to HCV infection are the main group of liver recipients in Western countries. As the infection usually recurs in the grafts, a noninvasive test for liver fibrosis is needed. In the literature, the usefulness of APRI in liver-transplanted patients with hepatitis C virus was evaluated and compared to other noninvasive tests: age-platelet index, aspartate aminotransferase to alanine aminotransferase ratio, Forns' fibrosis index, and Bonacini's discriminant

score. With AUROC of 0.81, APRI was the best test for discriminating patients with significant fibrosis (>2) in liver biopsy as compared to noninvasive methods mentioned above. The accuracy of APRI was higher in female recipients, with sensitivity of 91% and specificity of 75%, compared to 60% and 77% in men, respectively (Toniutto et al. 2007).

HIV-HCV Coinfection

HCV infection is a leading cause of morbidity in HIV-positive individuals, especially with the use of HAART and improvement of survival of HIV-infected patients (Singal et al. 2011). HIV-HCV coinfection is common in drug users as a result of shared routes of transmission.

The use of APRI in HIV-HCV coinfected patients was studied in fewer studies. Additionally, their limitations were small sample size and well-controlled HIV in the participants. The APRI performed better for biopsy size of ≥15 mm than ≥10 mm (AUROC for significant fibrosis 0.8 vs. 0.73, for cirrhosis 0.79 vs. 0.77, respectively) (Macias et al. 2006). According to Kelleher et al., AUROC was 0.71 in HIV/HCV coinfected patients, and the authors confirmed the need for liver biopsy size larger than 10 mm. The APRI predicted significant fibrosis with 91% certainty. Nine percent of patients were misclassified with high APRI (1.5) and F0 to F1 stage of liver fibrosis on the biopsy. A total of 27–34% of the patients could be potentially excluded from liver biopsy and treated for HCV. To sum up, the diagnostic accuracy of APRI was lower in coinfection HIV/HCV than in HCV monoinfection, which is in line with a previous meta-analysis (Mata-Marin et al. 2009).

The APRI of >1.5 was validated in adults with HIV-HCV coinfection as evidence of significant fibrosis, with high specificity but low sensitivity (Al-Mohri et al. 2005; Nunes et al. 2005; Kelleher et al. 2005). However, the best cutoff of APRI in pediatric population has not been established.

In a group of 1,012 perinatally HIV-infected Latin American children, the median of APRI was 0.29 (range, 0.05–29.67) and was elevated to >1.5 in 3.2% of patients (95% CI: 2.2–4.4%). There were factors associated with APRI >1.5: younger age, HBV coinfection, higher activity of alanine aminotransferase, lower concentration of total cholesterol, higher $\log_{10}$ current viral load, lower current CD4 count, lower nadir CD4 count, and the use of hepatotoxic non-antiretroviral medications. HCV infection did not increase the risk of APRI elevation.

Children with APRI of ≤1.5 had a better controlled HIV infection than patients with elevated APRI. Effective HIV treatment with HAART appeared to be beneficial for the liver, with a reduction in APRI, but long-term use of toxic non-antiretroviral drugs and comorbidities could have had an influence on liver outcome. A higher proportion of children with APRI of ≤1.5 experienced prior non-antiretroviral medications use compared to children with elevated APRI (85.3% vs. 65.6%; $p = 0.0053$) (Siberry et al. 2014). The prevalence of APRI >1.5 in a cohort of 451 perinatally HIV-infected children in the United States was 0.8% and was lower than in the previously mentioned cohort of children in Latin America. It could be due

to lower rates of other factors that contribute to liver damage, like other viral hepatitis or alcohol use. In both pediatric studies, longer antiretroviral treatment decreased the risk of APRI elevation.

Chronic Hepatitis B

The usefulness of noninvasive markers for significant liver fibrosis in patients with chronic hepatitis B (CHB) is not well established. Some authors found APRI as an accurate marker of fibrosis, but a few studies suggest that APRI may be of lower accuracy in liver fibrosis due to hepatitis B. It may be explained by wider regenerative nodules in CHB, more severe and less localized piecemeal necrosis, and fluctuating course with acute attacks of hepatitis B. Significant fibrosis was defined as stage ≥ 2 in liver biopsy according to the METAVIR system. In CHC, progression of fibrosis is more latent and piecemeal necrosis less localized. The AUROC of APRI in CHB patients in predicting fibrosis ranges from only 0.63 (Wai et al. 2006) and 0.708 (Seto et al. 2011) to 0.86 (Shin et al. 2008). A diagnostic tool is considered as good if AUROC is greater than 0.8. In the study of 89 HBV-infected patients, the APRI score was significantly higher in cirrhotic patients than in non-cirrhotic patients and was higher in significant fibrosis, but not statistically significant. The accuracy of the APRI score to determine cirrhosis was 72%, and the optimum APRI score cutoff point to identify such patients was 1.01 (Celikbilek et al. 2013). In another study, the value of APRI in identifying significant fibrosis and cirrhosis in HBV-infected patients was also limited, with AUROC of 0.62 and 0.67 for fibrosis and cirrhosis, respectively (Jin et al. 2012). In a recent meta-analysis, by Jin et al., AUROC of 0.79 and 0.75 was found. Some authors emphasize that APRI may be useful in the prediction of the absence of both cirrhosis and significant fibrosis, with a negative predictive value of over 90% in this group of patients.

To conclude, the APRI score did not seem to be as effective in determining fibrosis and cirrhosis as in CHC patients.

In children, such data are even more limited. Chronic HCV and HBV infections remain a rare problem in pediatric population, especially due to HBV vaccinations. But the pediatric population still needs a noninvasive marker of fibrosis to control treatment efficacy without liver biopsy, even more than adult population. Children usually require general anesthesia for liver biopsy and prolonged hospital stay to assess potential complications. McGoogan et al. evaluated APRI in predicting fibrosis and cirrhosis of the liver in 36 children with HCV or HBV infection compared to liver biopsy. The median APRI was 0.44 (0.24–0.97) in the group with HBV infection and 0.33 (0.20–0.44) among patients with hepatitis C. The area under the receiver operator characteristic curves was 0.71 for fibrosis and 0.52 for cirrhosis on liver biopsy. The authors concluded that APRI was moderately useful in predicting fibrosis in that group of children and could be a substitute for liver biopsy with AUC of 0.71 (McGoogan et al. 2010). Lebensztejn et al., in a group of 63 children with chronic hepatitis B virus, found an AUC of 0.74 and the highest

sensitivity and specificity of APRI for a cutoff of 0.59 (76.5% and 70%, respectively) (Lebensztejn et al. 2005).

Alcoholic Liver Disease (ALD)

The risk of end-stage liver disease in ALD increases with cumulative alcohol intake, but only minority of heavy drinkers suffer from advanced disease of the liver. The relationship between alcohol consumption and progression of liver injury in patients with and without HCV infection using APRI was evaluated in 1,308 patients. The sensitivity and specificity of APRI for significant fibrosis in HCV-positive patients (10.2%) were low: 35.6% and 29.7%, respectively. In HCV-negative patients ($N = 507$), the sensitivity of APRI for significant fibrosis was 13.2% and the specificity was 77.6%. In those groups of patients, APRI had a limited value in the diagnosis of fibrosis (Lieber et al. 2006). Heavy alcohol intake affects both AST and platelet count independently to the development of liver fibrosis. The role of alcohol in liver disease progression among HIV-infected patients was not clear. Hazardous drinking was found to be associated with increased APRI in a group of 1,358 HIV-infected patients, and 11.6% of them had APRI of >1.5. Viral hepatitis, male gender, and injection drug use as an HIV transmission route were other factors that predisposed to increased APRI. But among coinfected patients, increased APRI was found in 18.3%, and association with hazardous drinking was not confirmed. The authors defined minimal liver disease as APRI <0.4, significant liver disease as APRI >1.5, and 40 U/L as the upper limit of normal for AST (Chaudhry et al. 2009).

Nonalcoholic Fatty Liver Disease (NAFLD)

NAFLD seems to be the growing problem in industrialized countries. Liver fibrosis is one of the symptoms of nonalcoholic fatty liver disease. The wide spectrum of NAFLD symptoms ranges from simple steatosis, steatohepatitis, to liver cirrhosis. In adult patients with NAFLD, APRI had lower sensitivity than other noninvasive parameters: BARD, FIB-4 (age $\times$ AST level/platelet count $\times$ $\sqrt{}$ALT), NAFLD-fibrosis score and FibroMeter scores for advanced fibrosis (70% vs. 70.0%, 73.3%, 70.0%, 66.7%, and 66.7%, respectively), and higher specificity (74.5% vs. 66.4, 71.8%, 71.8%, and 74.5%, respectively). But no significant differences in sensitivity, specificity, or AUROCs were evident for those five scores in the diagnosis of advanced fibrosis (Subasi et al. 2015). Other studies demonstrated that such simple baseline noninvasive scores as APRI, NAFLD-fibrosis score, FIB-4 score, and BARD score can identify patients with an increased risk of liver-related complications and death. Among them, the NAFLD-fibrosis score was the most accurate, based on the area under the ROC curve and the analysis separating patient risk for a long-term outcome (Angulo et al. 2013).

Pediatric NAFLD is a different entity than NAFLD in adults. In children, histopathologic findings revealed portal inflammation and fibrosis rather than lobular

inflammation, ballooning, Mallory's hyaline, or perisinusoidal fibrosis. Among the hepatic fibrosis scores showing promising results in adult patients, only APRI and FIB4 revealed statistically significant differences between patients with mild and significant fibrosis. These two indexes might be regarded as useful methods to evaluate liver fibrosis in pediatric patients with NAFLD, while no single clinical or laboratory parameter can reflect the presence or severity of fibrosis in these patients (Yang et al. 2012). A recent study proved that FIB-4 index was a poor predictor of fibrosis and only APRI showed promising results for predicting the presence of any fibrosis with AUROC of 0.8 (Mansoor et al. 2015).

Retrospective studies assessed diagnostic ability of APRI for prediction of liver fibrosis compared to liver biopsy graded using the METAVIR scale (Beddosa and Poynard 1996) in viral chronic hepatitis or the Kleiner system for nonalcoholic fatty liver disease (Kleiner et al. 2005). Yilmaz et al. showed in 2011 that APRI was significantly associated with fibrosis scores in patients with NAFLD and chronic hepatitis C, but not in patients with chronic hepatitis B. In patients with CHC, APRI showed a higher sensitivity (72.7% vs. 60%) and lower specificity (62.4% vs. 73.3%), compared to NAFLD, respectively. The accuracy of APRI for the assessment of fibrosis score of 1–4 in patients with chronic hepatitis B was not acceptable with sensitivity of 55% and specificity of 75.4% (Yilmaz et al. 2011). According to the authors, the sensitivity of APRI depends on the etiology of liver injury. In case of NAFLD, APRI very rare reach value of more than 1. In such a condition, patients usually present mild to moderate increases in aminotransferases activity, and that is why APRI is elevated only in advanced stages of fibrosis as a result of gradual increases of AST in the presence of normal platelet counts. Besides, one should remember that alcohol consumption might have confounded association between APRI and stage of fibrosis, and it can depend on established maximal cutoff value of alcohol consumption (50 g/day for men and 30 g/day for women vs. 30 g/day for men and 20 g/day for women) to have a diagnose of nonalcoholic fatty liver disease (Loaeza-del-Castillo et al. 2008).

Primary Biliary Cirrhosis

Primary biliary cirrhosis (PBC) is an autoimmune slowly progressing cholestatic disease, in which outcome is largely dictated by development of cirrhosis. Up to one-third of patients treated with ursodeoxycholic acid (UDCA) do not show biochemical response. Patients not responding to UDCA treatment had a poorer outcome. APRI was related to histological progression in liver biopsy samples, and APRI of >0.54 at diagnosis may predict progression to liver failure. Elevated APRI is also associated with a risk of adverse events independently of treatment. However, compared with transient elastography (TE), APRI showed a low performance in diagnosing significant fibrosis. According to some authors, APRI at baseline and after 1 year following therapy may be an additive tool in identifying patients at risk of adverse outcome (Trivedi et al. 2014; Joshita et al. 2014).

Autoimmune Hepatitis

There were no correlations between the APRI score and stages of liver fibrosis in patients with autoimmune hepatitis (AIH). For the diagnosis of significant fibrosis (METAVIR $\geq$2) and cirrhosis (METAVIR 4), APRI values delimited an AUC of 0.602 and 0.599. In AIH, the portal, periportal, and lobular inflammation are observed in all stages of fibrosis. High AST levels are due to recurrent exacerbations and the impact of immunosuppressive treatments, irrespective of fibrosis stage. Both factors affect the value of APRI (Loaeza-del-Castillo et al. 2008).

Alpha-1-Antitrypsin Deficiency

Liver fibrosis is often seen in patients with α-1-antitrypsin deficiency (ATD), and in the literature, it was found to be an indicator of bad outcome in this group of patients. As the groups of patients with ATD are small, there are data from one study concerning APRI effectiveness in liver fibrosis due to alpha-1-antitripsin deficiency. In the group of 21 Polish children with ATD, APRI was 0.22 (0.12–0.39), median (Q1-Q3). For advanced fibrosis, the optimal cutoff value for APRI was 0.26 while for cirrhosis – 0.33. The sensitivity was low, i.e., 0.60 (95%CI 0.41–0.77) and 0.83 (0.36–0.99), and the specificity was 0.87 (95%CI 0.60–0.98) and 0.31 (0.17–0.48), respectively, for advanced fibrosis and cirrhosis. The usefulness of APRI in detection of liver cirrhosis (AUROC 0.51) in alpha-1-AT deficiency in children was assessed as doubtful (Bakula et al. 2012).

Biliary Atresia

APRI was also investigated in infants with biliary atresia. It was found to be effective in diagnosing significant liver fibrosis, especially cirrhosis at presentation with the area under the receiver operator characteristic curve of 0.75 and 0.81, respectively. APRI of >0.60 before the Kasai procedure could predict jaundice persistence but neither occurrence of cholangitis after surgery nor development of esophageal varices. In cases with highly suspected biliary atresia, APRI may decrease the need for liver biopsy (Yang et al. 2015). APRI was correlated with age, size of spleen, and bilirubin concentration. Survival with a native liver was better for patients with the lowest APRI (Grieve et al. 2013).

APRI was proposed as the first-line test in the diagnostic approach named SAFE (sequential algorithm for fibrosis evaluation) biopsy algorithm in patients with chronic hepatitis B and C (Sebastiani et al. 2007). The aim of the algorithm was to reduce the number of liver biopsies. According to this algorithm, the biopsy was used as the third-line test in cases of no enough accuracy of APRI and Fibrotest. Fibrotest combines total bilirubin, GGTP, haptoglobin, alpha-2-macroglobulin, apolipoprotein A1, age, and gender. To date, it is validated in viral hepatitis B and C coinfection, HIV/HCV, ALD, and NAFLD. In the SAFE biopsy algorithm in

significant fibrosis (METAVIR≥F2), APRI of ≤0,5 had a low NPV, and such patients could not avoid liver biopsy. There was no need of biopsy when APRI was higher than 1.5. In the range between 0.5 and 1.5, further decision was dependent on Fibrotest: >0.49 confirmed the presence of significant fibrosis without the need of liver biopsy. The 1 cutoff of APRI excluded cirrhosis, while 2 cutoff together with fibrotest ≥0.75 confirmed cirrhosis. Combination of algorithms could help in monitoring of both, disease progression and antiviral therapies. APRI was also modified as Lok index with improvement of the diagnostic accuracy: alanine aminotransferase (ALT) and international normalized ratio (INR) were added (Lok et al. 2005).

APRI and Lok index were investigated with other noninvasive parameters of liver fibrosis in predicting the presence of esophageal varices (OV) in cirrhotic patients. But neither these simple tests nor fibroscan could predict the presence of OV and replace endoscopy with screening of esophageal varices (Sebastiani et al. 2010).

Summary Points

1. The diagnostic usefulness of the APRI score depends on the etiology of chronic liver injury.
2. The APRI score correlates significantly with fibrosis stage in patients with chronic hepatitis C.
3. It does not seem to have a diagnostic value in patients with autoimmune hepatitis, chronic hepatitis B, or alcoholic liver disease.
4. APRI showed promising results for predicting the presence of any fibrosis in pediatric patients with NAFLD.
5. Compared with transient elastography, which is a new, noninvasive, painless, and objective method, APRI showed low performance in diagnosing significant fibrosis.
6. Neither APRI nor fibroscan could predict the presence of OV and replace endoscopy with screening of OV.

References

Al-Mohri H, Cooper C, Murphy T, et al. Validation of a simple model for predicting liver fibrosis in HIV/hepatitis C virus-coinfected patients. HIV Med. 2005;6:375–8.

Angulo P, Bugianesi E, Bjornsson ES, et al. Simple noninvasive systems predict long-term outcomes of patients with nonalcoholic fatty liver disease. Gastroenterology. 2013;145:782–9.

Bakula A, Dadalski M, Pronicki M, et al. Apri as an indicator of advanced liver fibrosis in children with alpha-1-antitrypsin deficiency. Prz Gastroenterol. 2012;7:379–82.

Beddosa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology. 1996;24(2):289–93.

Celikbilek M, Dogan S, Gursoy S, et al. Noninvasive assessment of liver damage in chronic hepatitis B. World J Hepatol. 2013;5:439–45.

Chaudhry AA, Sulkowski MS, Chander G, et al. Hazardous drinking is associated with an elevated aspartate aminotransferase to platelet ratio index in an urban HIV-infected clinical cohort. HIV Med. 2009;10:133–42.

Fattovich G, Giustina G, Degos F, et al. Morbidity and mortality in compensated cirrhosis type C: retrospective follow-up study of 384 patients. Gastroenterology. 1997;112:463–72.

Grieve A, Makin E, Davenport M. Aspartate Aminotransferase-to-Platelet ratio index (APRi) in infants with biliary atresia: prognostic value at presentation. J Pediatr Surg. 2013;48: 789–95.

Jin W, Lin Z, Xin Y, et al. Diagnostic accuracy of the aspartate aminotransferase-to-platelet ratio index for the prediction of hepatitis B-related fibrosis: a leading meta-analysis. BMC Gastroenterol. 2012;12:14. doi:10.1186/1471-230X-12-14. PMID:22333407.

Joshita S, Umemura T, Ota M, et al. AST/platelet ratio index associates with progression to hepatic failure and correlates with histological fibrosis stage in Japanese patients with primary biliary cirrhosis. J Hepatol. 2014;61:1443–5.

Kelleher TB, Mehta SH, Bhaskar R, et al. Prediction of hepatic fibrosis in HIV/HCV co-infected patients using serum fibrosis markers: the SHASTA index. J Hepatol. 2005;43:78–84.

Kim SY. Noninvasive markers for the diagnosis of nonalcoholic Fatty liver disease. Endocrinol Metab (Seoul). 2013;28(4):280–2.

Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41:1313–21.

Lebensztejn DM, Skiba E, Sobaniec-Lotowska M, Kaczmarski M. A simple noninvasive index (APRI) predicts advanced liver fibrosis in children with chronic hepatitis B. Hepatology. 2005;41:1434–5.

Lieber CS, Weiss DG, Morgan TR, et al. Aspartate aminotransferase to platelet ratio index in patients with alcoholic liver fibrosis. Am J Gastroenterol. 2006;101:1500–8.

Lin ZH, Xin YN, Dong QJ, et al. Performance of the aspartate aminotransferase-to-platelet ratio index for the staging of hepatitis C-related fibrosis: an updated meta-analysis. Hepatology. 2011;53:726–36.

Loaeza-del-Castillo A, Paz-Pineda F, Oviedo-Cardenas E, et al. AST to platelet ratio index (APRI) for the noninvasive evaluation of liver fibrosis. Ann Hepatol. 2008;7:350–7.

Lok AS, Ghany MG, Goodman ZD, et al. Predicting cirrhosis in patients with hepatitis C based on standard laboratory tests: results of the HALT-C cohort. Hepatology. 2005;42:282–92.

Macias J, Giron-Gonzalez JA, Gonzalez-Serrano M, et al. Prediction of liver fibrosis in human immunodeficiency virus/hepatitis C virus coinfected patients by simple non-invasive indexes. Gut. 2006;55:409–14.

Mansoor S, Yerian L, Kohli R, et al. The evaluation of hepatic fibrosis scores in children with nonalcoholic fatty liver disease. Dig Dis Sci. 2015;60:1440–7.

Mata-Marin JA, Fuentes-Allen JL, Gaytan-Martinez J, et al. APRI as a predictor of early viral response in chronic hepatitis C patients. World J Gastroenterol. 2009;15:4923–7.

McGoogan KE, Smith PB, Choi SS, et al. Performance of the AST to Platelet Ratio Index (APRI) as a noninvasive marker of fibrosis in pediatric patients with chronic viral hepatitis. J Pediatr Gastroenterol Nutr. 2010;50:344–6.

Mummadi RR, Petersen JR, Xiao SY, et al. Role of simple biomarkers in predicting fibrosis progression in HCV infection. WJG. 2010;16:5710–5.

Naveau S, Gaudé G, Asnacios A, Agostini H, Abella A, Barri-Ova N, Dauvois B, Prévot S, Ngo Y, Munteanu M, Balian A, Njiké-Nakseu M, Perlemuter G, Poynard T. Diagnostic and prognostic values of noninvasive biomarkers of fibrosis in patients with alcoholic liver disease. Hepatology. 2009;49(1):97–105.

Ngo Y, Munteanu M, Messous D, Charlotte F, Imbert-Bismut F, Thabut D, Lebray P, Thibault V, Benhamou Y, Moussalli J, Ratziu V, Poynard T. A prospective analysis of the prognostic value of biomarkers (FibroTest) in patients with chronic hepatitis C. Clin Chem. 2006;52(10): 1887–96.

Nguyen-Khac E, Chatelain D, Tramier B, Decrombecque C, Robert B, Joly JP, Brevet M, Grignon P, Lion S, Le Page L, Dupas JL. Assessment of asymptomatic liver fibrosis in alcoholic patients using fibroscan: prospective comparison with seven non-invasive laboratory tests. Aliment Pharmacol Ther. 2008;28(10):1188–98.

Nunes D, Fleming C, Offner G, et al. HIV infection does not affect the performance of noninvasive markers of fibrosis for the diagnosis of hepatitis C virus related liver disease. J Acquir Immune Defic Syndr. 2005;40:538–44.

Sebastiani G, Vario A, Guido M, et al. Sequential algorithms combining non-invasive markers and biopsy for the assessment of liver fibrosis in chronic hepatitis B. World J Gastroenterol. 2007;13:525–31.

Sebastiani G, Tempesta D, Fattovich G, et al. Prediction of oesophageal varices in hepatic cirrhosis by simple serum non-invasive markers: results of a multicenter, large-scale study. J Hepatol. 2010;53:630–8.

Seto W-K, Lee C-F, Lai C-L, et al. A new model using routinely available clinical parameters to predict significant liver fibrosis in chronic hepatitis B. PLoS One. 2011;6(8):e23077. doi:10.1371/journal.pone.0023077.

Shaheen AA, Myers RP. Diagnostic accuracy of the aspartate aminotransferase-to-platelet ratio index for the prediction of hepatitis C-related fibrosis: a systematic review. Hepatology. 2007;46(3):912–21.

Shaheen AA, Wan AF, Myers RP. FibroTest and FibroScan for the prediction of hepatitis C-related fibrosis: a systematic review of diagnostic test accuracy. Am J Gastroenterol. 2007;102:2589–600.

Shin WG, Park SH, Jang MK, et al. Aspartate aminotransferase to platelet ratio index (APRI) can predict liver fibrosis in chronic hepatitis B. Dig Liver Dis. 2008;40:267–74.

Siberry GK, Cohen RA, Harris DR, et al. Prevalence and predictors of elevated aspartate aminotransferase-to-platelet ratio index in Latin American perinatal HIV-infected children. Pediatr Infect Dis J. 2014;33:177–82.

Singal AG, Thomassen LV, Gretch DR, et al. Use of the AST to platelet ratio index in HCV/HIV co-infected patients. Aliment Pharmacol Ther. 2011;33:566–77.

Snyder N, Gajula L, Xiao SY, Grady J, Luxon B, Lau DT, Soloway R, Petersen J. APRI: an easy and validated predictor of hepatic fibrosis in chronic hepatitis C. J Clin Gastroenterol. 2006;40(6):535–42.

Sorrell MF, Belongia EA, Costa J, et al. National Institutes of Health Consensus Development Conference Statement: management of hepatitis B. Ann Intern Med. 2009;150:104–10.

Subasi CF, Aykut UE, Yilmaz Y. Comparison of noninvasive scores for the detection of advanced fibrosis in patients with nonalcoholic fatty liver disease. Eur J Gastroenterol Hepatol. 2015;27:137–41.

Toniutto P, Fabris C, Bitetto D, et al. Role of AST to platelet ratio index in the detection of liver fibrosis in patients with recurrent hepatitis C after liver transplantation. J Gastroenterol Hepatol. 2007;22:1904–8.

Trivedi PJ, Bruns T, Cheung A, et al. Optimising risk stratification in primary biliary cirrhosis: AST/platelet ratio index predicts outcome independent of ursodeoxycholic acid response. J Hepatol. 2014;60:1249–58.

Wai CT, Greenson JK, Fontana RJ, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology. 2003;38:518–26.

Wai CT, Cheng CL, Wee A, et al. Non-invasive models for predicting histology in patients with chronic hepatitis B. Liver Int. 2006;26:666–72.

Yang HR, Kim HR, Kim MJ, et al. Noninvasive parameters and hepatic fibrosis scores in children with nonalcoholic fatty liver disease. World J Gastroenterol. 2012;18:1525–30.

Yang LY, Fu J, Peng XF, et al. Validation of aspartate aminotransferase to platelet ratio for diagnosis of liver fibrosis and prediction of postoperative prognosis in infants with biliary atresia. World J Gastroenterol. 2015;21:5893–900.

Yilmaz Y, Yonal O, Kurt R, et al. Noninvasive assessment of liver fibrosis with aspartate transaminase to platelet ratio (APRI): usefulness in patients with chronic liver disease. Hepatol Mon. 2011;11:103–7.

Yu ML, Lin SM, Lee CM, Dai CY, Chang WY, Chen SC, Lee LP, Lin ZY, Hsieh MY, Wang LY, Chuang WL, Liaw YF. A simple noninvasive index for predicting long-term outcome of chronic hepatitis C after interferon-based therapy. Hepatology. 2006;44(5):1086–97.

Soluble CD163 (sCD163): Biomarker of Kupffer Cell Activation in Liver Disease

15

Holger Jon Møller, Konstantin Kazankov, Sidsel Rødgaard-Hansen, Marlene Christina Nielsen, Thomas D. Sandahl, Hendrik Vilstrup, Søren Kragh Moestrup, and Henning Grønbæk

Contents

H.J. Møller (✉) • S. Rødgaard-Hansen (✉) • M.C. Nielsen (✉)
Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark
e-mail: holgmoel@rm.dk; annthrse@rm.dk; sidsroed@rm.dk; machne@rm.dk

K. Kazankov (✉) • T.D. Sandahl (✉) • H. Vilstrup (✉) • H. Grønbæk (✉)
Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark
e-mail: konskaza@rm.dk; thomsand@rm.dk; vilstrup@clin.au.dk; henngroe@rm.dk

S.K. Moestrup (✉)
Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark
e-mail: smoestrup@health.sdu.dk

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_40

Abstract

Soluble CD163 (sCD163) is a specific plasma biomarker for macrophage activation. In liver disease, the marker reflects Kupffer cell activation during inflammation and oxidative stress, and it relates to disease severity, prognosis, and treatment responses in a number of inflammatory conditions affecting the liver.

Very high levels of sCD163, that closely relate to disease severity and outcome, are found in situations of overt inflammation and necrosis such as acute liver failure, acute-on-chronic liver failure, and alcoholic hepatitis. In liver cirrhosis the biomarker reflects disease severity. It associates with severity scores (MELD, Child-Pugh) and portal hypertension, and it accurately predicts disease progression and survival. Similarly, there is a stepwise increase in sCD163 with increasing inflammation and fibrosis in chronic hepatitis B and C, with diagnostic accuracy for significant fibrosis exceeding standard diagnostic scores such as APRI and FIB-4. Lower levels are found in nonalcoholic fatty liver disease (NAFLD), but sCD163 adds diagnostic information on the important identification of patients with advanced disease.

The emerging picture from the increasing number of publications related to sCD163 in liver disease unequivocally identifies sCD163 as a clinical useful biomarker that adds important information to diagnosis, prognosis, treatment, and monitoring of disease. The biomarker is currently measured by research-grade ELISA-assays. A safe introduction of decision limits and prognostic scores into clinical routine use requires an international standardization and preferably a transfer to automated analytical platforms.

Keywords

CD163 • sCD163 • Biomarker • Macrophage • Kupffer cell • ELISA • NAFLD • Hepatitis • Cirrhosis • Liver failure

List of Abbreviations

ACLF	Acute-on-chronic liver failure
ADAM	A disintegrin and metalloproteinase
AH	Alcoholic hepatitis
ALD	Alcoholic liver disease
ALF	Acute liver failure
ALP	Alkaline phosphatase
ALT	Alanine transaminase
APRI	AST to platelet ratio index
AST	Aspartate transaminase
AUC	Area under the curve
AUROC	Area under receiver operating characteristic
BCLC	Barcelona clinic liver cancer
CANONIC	CLIF acute-on-chronic liver failure
CD163	Cluster of differentiation 163
CP	Child-Pugh

CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
FIB-4	Fibrosis-4
GAHS	Glasgow alcoholic hepatitis score
GEC	Galactose elimination capacity
GGT	Gamma-glutamyl transferase
Hb	Hemoglobin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDL	High-density lipoprotein
HLH	Hemophagocytic lymphohistiocytosis
Hp	Haptoglobin
Hp-Hb	Haptoglobin-hemoglobin
HVPG	Hepatic venous pressure gradient
IL	Interleukin
INR	International normalized ratio
LBP	LPS binding proteins
LPS	Lipopolysaccharides
MELD	Model for end-stage liver disease
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
PAMP	Pathogen-associated molecular pattern
SIRS	Systemic inflammatory response syndrome
TAMs	Tumor-associated macrophages
TGF	Transforming growth factor
TIPS	Transjugular intrahepatic portosystemic shunt
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNM	Tumor nodes metastasis
TWEAK	Tumor necrosis factor-α-like weak inducer of apoptosis

Key Facts of the CD-System

- The CD (cluster of differentiation) system is a nomenclature for classification of proteins on the surface of cells (predominantly white blood cells).
- The proteins are assigned a CD-number at international conferences/workshops (first held in Paris 1982) primarily based on its reactivity with monoclonal antibodies.
- The CD-system is used as a reference for the identification of cells and for classifying cells into specific cell subtypes.
- The expression pattern of different CD-molecules on the cell surface can be measured by immunological techniques (e.g., flow cytometry or immunohistochemistry).

- The CD-molecules have different functions. Some, like CD163, act as receptors for the binding and internalization of specific molecules.
- CD163 was assigned its number at the VI workshop in Kobe 1996. It was later discovered to function as a receptor responsible for the removal of Hemoglobin-Haptoglobin complexes from the blood by macrophages.

Definitions of Words and Terms

Acute liver failure	Severe liver damage with critical illness and complications such as hepatic encephalopathy.
AUROC	Area under the receiver operating characteristic is a statistical method used, e.g., for assessing the diagnostic accuracy of a test (measured biomarker). An ideal test with no false negatives or positives will have an AUROC of 1.0.
Biological variation	In this context, the variation in the concentration of a biomarker in the blood between or within individuals.
ELISA, Enzyme Linked Immuno Sorbent Assay	A method for measuring the concentration of molecules (typically proteins) in a solution such as serum. Typically the protein is captured by a fixed antibody and hence detected by a labeled secondary antibody.
Hepatocellular carcinoma	The most common primary (i.e., not metastatic) cancer of the liver. It is often preceded by chronic viral hepatitis or liver cirrhosis.
Liver cirrhosis	The end stage of many chronic liver disease characterized by scarring of the liver and loss of liver cells.
Macrophage	An important cell type of the innate immune system performing essential tasks related to scavenging of cell debris and foreign particles, antigen recognition, and initiation of immune responses. The resident macrophages of the liver are termed Kupffer cells.
NAFLD. Nonalcoholic fatty liver disease	A disease of the liver (often related to obesity and life style), with accumulation of liver fat and in some cases progression to NASH, characterized by inflammation and liver fibrosis.
Portal hypertension	High blood pressure in the portal vein and its branches, which is often due to liver cirrhosis.

Scavenger receptor	Proteins on the cell surface (typically macrophages) that bind and internalize specific foreign or waste molecules for degradation inside the cell.

Introduction

History of sCD163 in Liver Disease

Since the discovery in 2001 of the important role of cluster of differentiation 163 (CD163) in the removal of hemoglobin (Hb) from plasma (Kristiansen et al. 2001), studies on the receptor function has increased our knowledge of iron-metabolism, inflammation, and not least macrophage biology. The macrophage-specific and strong expression of CD163 are unique features that are increasingly utilized for diagnostic purposes in pathology and related fields. Its endocytic properties are currently being evaluated as a target for specific direction of drugs to macrophages which may have a great potential for the treatment of inflammation and cancer (Etzerodt and Moestrup 2013).

It was quickly realized that a soluble form of the receptor circulates in high concentrations in plasma (soluble CD163, sCD163) and that this molecule may reflect macrophage activation in the tissues (Møller et al. 2002b). Since then an accelerating number of publications have used or evaluated the biomarker in various disease conditions where macrophages are involved (Møller 2012). Besides its extensive use in research, the marker is now entering routine clinical diagnostics in selected areas such as macrophage activation syndromes (hemophagocytic lymphohistiocytosis [HLH]) and Gaucher disease. The most important field of use seems, however, to be in hepatology, which is the focus of this review. During the last few years a large number of studies have unequivocally demonstrated important and relevant applications of the biomarker for diagnosis, prognosis, and disease monitoring in various liver diseases. In 2005 the first studies reporting on CD163 expression and sCD163 in liver diseases were related to viral hepatitis and acute liver failure (ALF) (Hiraoka et al. 2005b). Since then a number of studies have reported on macrophage activation and elevated sCD163 levels in patients with ALF (Møller et al. 2007), acetaminophen intoxication (Craig et al. 2013), chronic liver disease and portal hypertension (Holland-Fischer et al. 2011), chronic viral hepatitis B and C (Andersen et al. 2014; Kazankov et al. 2014), alcoholic hepatitis (AH) (Sandahl et al. 2014), hepatocellular carcinoma (HCC) (Waidmann et al. 2013b), and nonalcoholic fatty liver disease (NAFLD) (Kazankov et al. 2015b) with an increasing number of publications during the last years.

sCD163 is currently measured using in-house or commercially available research assays, helped along by the high stability of the protein and the relatively high concentrations found in the blood. An outstanding challenge, however, is the lack of assay standardization resulting in the reporting of varying levels across studies, a topic that needs to be solved before the biomarker can be used for critical decision-making in the routine clinic.

The Kupffer Cell and Inflammatory Liver Diseases

Macrophages are key players in both liver homeostasis and in the development of inflammatory liver disease. Kupffer cells, the resident macrophages of the liver, constitute the largest population of tissue macrophages in the body, reportedly up to 80–90%. Liver injury results in additional recruitment of circulating monocytes, which differentiate into tissue macrophages in the liver. Furthermore, there is evidence to suggest that also the resident Kupffer cells can multiply and thus contribute to the accumulation of macrophages (Tacke and Zimmermann 2014).

Phagocytosis, the ingestion of cells, cell fragments, microorganisms, or foreign particulates for lysosomal degradation, is an essential macrophage characteristic. Due to their location on the luminal side of the liver sinusoid, the Kupffer cells are continuously exposed to exogen material absorbed from the intestinal tract and serve as a first line defense (Tacke and Zimmermann 2014).

Irrespective of underlying pathology, hepatocyte death is a driving force in the initiation and propagation of hepatic inflammation. Hepatocyte bodies stimulate the production of inflammatory cytokines from the macrophages. Kupffer cells express toll-like receptors (TLRs) which recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide and bacterial DNA. Stimulation through TLR-4 promotes pro-inflammatory polarization with increased production of tumor necrosis factor (TNF)-α, a pleiotropic inflammatory cytokine, which in turn activates the hepatic stellate cells and further their survival (Ilan 2012; Eckert et al. 2015; Seki and Schwabe 2015). Hepatic stellate cells then turn into myofibroblasts which produce increased amounts of extracellular matrix causing liver fibrosis and reduced liver function.

The macrophage population is characterized by a remarkable heterogeneity. Traditionally, macrophages have been classified as either M1 or M2 types. M1 macrophages denote the pro-inflammatory subtype, whereas M2 macrophages are considered immunoregulatory and anti-inflammatory. However, it is now accepted that the simplistic M1/M2 model does not adequately describe the diversity of the macrophage cell population (Martinez and Gordon 2014). During inflammation the Kupffer cells produce cytotoxic substances such as reactive oxygen radicals causing liver necrosis, and in this phase macrophage depletion dampens fibrosis progression. Conversely, during fibrosis resolution the depletion of macrophages weakens the matrix degradation and prevents recovery. This suggests that the macrophage have dual, opposing functions in liver pathology both promoting and attenuating inflammation and fibrosis (Tacke and Zimmermann 2014).

CD163: The Hemoglobin Receptor

CD163 is a 130 kDa monocyte/macrophage protein (Pulford et al. 1992; Law et al. 1993) functioning as the scavenger receptor of the tight complex of haptoglobin (Hp) and Hb (Hp-Hb) formed instantly in plasma when Hb escapes red cells during intravascular hemolysis (Kristiansen et al. 2001). CD163 has also been reported to bind human pathogenic bacteria (Fabriek et al. 2009), tumor necrosis factor-α-like weak inducer of apoptosis (TWEAK) (Bover et al. 2007), and certain pig-pathogenic virus strains (Sanchez-Torres et al. 2003; Bover et al. 2007; Van Gorp et al. 2008).

CD163 consists of nine scavenger receptor protein modules, a transmembrane segment, and a short C-terminal cytoplasmic tail (Fig. 1). The scavenger receptor protein modules, which represent the entire extracellular domain of the receptor, are responsible for ligand binding and the cytoplasmic tail signals the endocytosis activity of the receptor. The endocytic function of CD163 parallels that of the canonical low-density lipoprotein receptor. In accordance with classical endocytosis, CD163 constitutively recycles between the plasma membrane, where it picks up the ligand, and endosomes, where it releases it again. A decrease in pH and the calcium concentration in the endosomes are responsible for the release of the cargo that subsequently is transported to the lysosomal degradation pathway (Andersen and Moestrup 2014).

This endocytic activity of CD163 and its high affinity for Hp-Hb, but not for circulating Hp without Hb, explains the depletion of Hp during hemolytic diseases. During physiological intravascular hemolysis that causes degradation of approximately 0.5–1 g Hb per day, the production of Hp (mainly in the hepatocytes) is in equilibrium with the degradation. All human Hp phenotypes (Hp 1–1, 2–1 and 2–2)

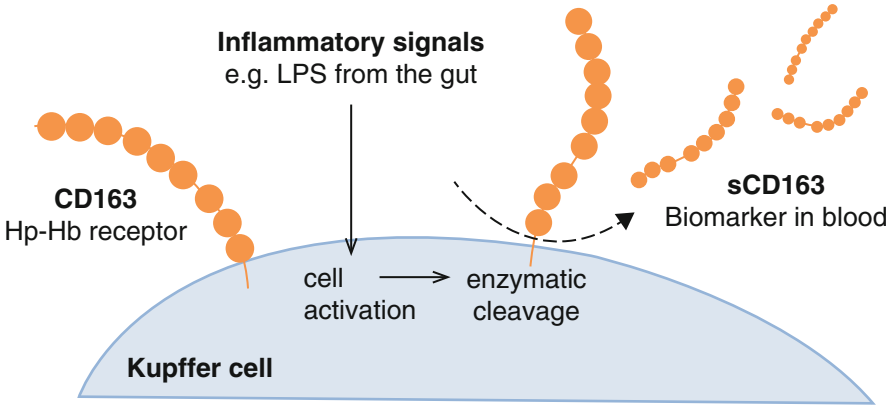

Fig. 1 Shedding of sCD163 by inflammatory signals. The macrophage-specific CD163 protein functions as the Haptoglobin-Hemoglobin (Hp-Hb) receptor (Kristiansen et al. 2001). Inflammatory signals induce shedding of soluble CD163 (sCD163) which works as a specific biomarker reflecting macrophage involvement in disease

bind with high affinity to CD163 when complexed to Hb. The degradation of Hb leads to the conversion of heme to bilirubin, iron, and CO. Bilirubin is released to the plasma where it by means of albumin is transported to the hepatocytes for conjugation and secretion into the bile.

CD163 expression is highly regulated, and in vitro studies have shown upregulation by heme, Hb, glucocorticoids, interleukin (IL)-6, and IL-10, whereas lipopolysaccharide (LPS), TNF, IL-4, and granulocyte-macrophage colony-stimulating factor downregulate expression (Etzerodt and Moestrup 2013). Moreover, CD163 is highly upregulated during maturation of macrophages from monocytes, which have a low expression. Tissue macrophages of the M2 type with high CD163 expression are, for instance, seen in the bone marrow, spleen red pulp, and liver.

Soluble CD163

A soluble form of CD163, sCD163 is present in plasma and other body fluids, at least partly due to proteolytic shedding of the receptor from monocytes and macrophages (Droste et al. 1999; Sulahian et al. 2001). The protein is constitutively released from the cells; however, in case of macrophage activation, the concentration increases, and therefore sCD163 works as a specific biomarker reflecting macrophage involvement in disease (Møller 2012).

A number of inflammatory stimuli are known to result in sCD163 shedding in vitro, including bacterial endotoxin (LPS), oxidative stress, and thrombin (Møller 2012) (Fig. 1). One specific mechanism of LPS-mediated shedding was described by Etzerodt et al. (2010) who showed the dependence of a disintegrin and metalloproteinase (ADAM) 17, which also cleaves pro-TNF-a, in this process. In acute sepsis, and after experimental LPS injection, the concentration of sCD163 rises quickly approximately four times. This increase is, however, far from the $>$10 times increase observed in patients during cytokine storms in macrophage activation syndromes and ALF (Hintz et al. 2002; Schaer et al. 2005; Møller et al. 2007; Etzerodt et al. 2010). Other mechanisms of release may therefore be involved.

The circulating sCD163 consists of almost the entire extracellular domain (Møller et al. 2010), including the region shown to bind hemoglobin-haptoglobin complexes. The soluble form, however, binds considerably weaker to the complexes than the membrane-bound form, possibly due to multivalent binding on the cell membrane. The physiological functions of sCD63 are not known, but are probably part of the innate immune defense. The molecule has been shown to bind *Staphylococcus aureus* and mediate a fast clearance from plasma of the bacteria (Kneidl et al. 2012).

Due to the importance of macrophages in inflammatory diseases, there is an increasing focus on more macrophage-specific inflammatory markers, and sCD163 has in recent years been evaluated for its diagnostic and prognostic value in a number of diseases such as HIV and rheumatoid arthritis and has shown to be especially valuable in liver disease.

Kupffer Cell Activation and sCD163

The cellular origin of the plasma sCD163 in the normal and diseased states is not known. CD163 is expressed on macrophages in a number of tissues, and also monocytes express CD163, although at lower levels than macrophages. When liberated from the monocytes/macrophages, sCD163 has a half-life of ½–1 day, which makes it difficult to assess the tissue origin. A significant part of the circulating sCD163, however, probably derives from Kupffer cells. These cells constitute the majority of macrophages in the body, they are located in the blood-stream, and CD163 is significantly upregulated on the Kupffer cells in inflammatory liver diseases. Furthermore, there are clear experimental indications to support this. In a study of cirrhosis patients, the concentration of sCD163 in the hepatic vein was 12% higher than in the portal vein (Holland-Fischer et al. 2011). Higher hepatic vein concentrations were also seen in ALF patients (Antoniades et al. 2013), and 23% higher hepatic vein concentrations were seen in obese/NAFLD patients (Kazankov et al. 2015c). A small study in liver-healthy individuals did not show a difference over the liver (Bauer et al. 2011).

Aspects of Measuring sCD163

The concentration of sCD163 in plasma is in the low mg/L level, and the protein is easily measured by enzyme-linked immunosorbent assays (ELISA) with good precision. No CE-labeled assays or kits for automated platforms have been launched yet. The ELISA format is well suited for research samples; however, it is less practical for measuring few routine samples on a daily basis. A number of commercial assays are now available of which the Macro163® kit from IQ-products (formerly Trillium) and the CD163 Quantikine ELISA Kit from R&D have most widely been used in published studies on liver disease. Many studies, however, have been performed using an in-house assay developed at the department of Clinical Biochemistry in Aarhus (Møller et al. 2002a). The three assays correlate strongly; however, the level measured by the two commercial assays are lower than obtained by the in-house assay, amounting to approximately 40% in one comparison study (Møller 2012). Until a thorough standardization is performed, reference ranges and clinical decision limits need to be adjusted according to the assay. Of great advantage is the stability of the protein in serum. Constant levels are found in samples that have been stored for several years at −80 °C, and samples can be frozen and thawed repeatedly without loss of immune reactivity (Møller et al. 2002a). Some assays may be sensitive to EDTA in plasma or hemolysis (Maniecki et al. 2011).

Concentration in Healthy Individuals and Biological Variation of sCD163

The concentration of sCD163 in healthy individuals (95% reference range based on 240 healthy Scandinavians) is 0.7–3.8 mg/L using the Aarhus assay (Møller 2012),

with median levels of 1.7 mg/L. Levels increase slightly with age (Møller et al. 2003). Similar levels were found when screening 9000 individuals from the general population (Møller et al. 2011). The mean concentration in smaller, healthy control groups used in the published studies on liver disease ranged from 1.5 to 2.3 mg/L using the in-house assay (Møller et al. 2007; Holland-Fischer et al. 2011; Grønbæk et al. 2012; Rode et al. 2013; Sandahl et al. 2014) and from 0.4 to 0.8 mg/L using commercial assays (Craig et al. 2013; Ye et al. 2013; Sprinzl et al. 2015).

Whereas there is a large interindividual variation in the concentration of sCD163, the concentration within the individual is remarkably stable over time. This is true for healthy individuals (Møller et al. 2003, 2011) but also for patients with stable liver disease (Waidmann et al. 2013a). This is important since sCD163 will be especially useful for the monitoring of patient disease and intervention. Until now most studies on sCD163 in liver disease have focused on diagnostic and prognostic aspects of liver disease; however, it is very possible that sCD163 will prove to be a valuable biomarker for the follow-up of the course of disease in liver patients. Based on a total analytical error of 6% and an intraindividual biological variation of 9% (Møller et al. 2003), it can be estimated that a >30% change between two sCD163 measurements over time is clinically significant.

In healthy individuals and in the general population there is a weak but highly significant correlation between sCD163 and liver parameters (alanine transaminase (ALT), gamma glutamyl transferase (GGT)), and metabolic parameters (BMI, Triglycerides, Cholesterol (total and high-density lipoprotein (HDL))) (Møller et al. 2011; Møller 2012).

Soluble CD163 in Liver Disease

sCD163 in Nonalcoholic Fatty Liver Disease (NAFLD)

Due to the close association with obesity, there is an epidemic increase in the prevalence of NAFLD, which is now an emerging healthcare problem and a steady increasing indication for liver transplantation (Vernon et al. 2011). sCD163 is slightly elevated in obesity and shows a clear association to both traditional liver biochemistry (e.g., ALT), lipids (e.g., triglycerides), and low-grade inflammation (C-reactive protein [CRP]) (Parkner et al. 2012; Fjeldborg et al. 2013). In addition, sCD163 is associated with insulin resistance (Parkner et al. 2012; Zanni et al. 2012; Kracmerova et al. 2014). Although the increase seen in apparently healthy obese individuals is subtle, these changes seem to be able to predict later development of diabetes and liver disease (Møller et al. 2011, 2012).

The multifactorial pathogenesis of NAFLD and its more severe manifestation nonalcoholic steatohepatitis (NASH) includes innate immunity and its central regulators macrophages. Experimental studies of different animal models suggest crucial involvement of liver-resident (Kupffer cells) and recruited macrophages in the development of steatosis, inflammation, and fibrogenesis (Tomita et al. 2006; Stienstra et al. 2010). In NAFLD, macrophages can be activated by different stimuli.

The two best-described mechanisms are activated through bacterial translocation/endotoxin (Ye et al. 2012), and by certain lipids and their metabolites (Bohm et al. 2013; Miura et al. 2013), however, other mechanisms have been described. Macrophages exert their actions through the production of pro-inflammatory cytokines such as TNF-α (Tomita et al. 2006), IL-1β (Stienstra et al. 2010), IL-6, and transforming growth factor (TGF)-β (Kodama et al. 2009). Furthermore, the macrophages are instrumental for the recruitment of B and T lymphocytes, and they interact with natural killer T cells, and contribute to oxidative stress.

Macrophage activation in NAFLD is reflected by an increase in CD163 expression. In a study of children with biopsy-proven NAFLD and NASH, CD163+ cells were markedly increased in those with severe histological activity (NAS $\geq$ 5) compared to those with lower activity, and the CD163+ cells were also associated with the presence of fibrosis (De Vito et al. 2012). In adults with NASH, we observed a distinct distribution of CD163+ macrophages, forming microgranulomas and aggregation around steatotic hepatocytes. Some CD163+ macrophages contained lipid droplets (Kazankov et al. 2015c).

Soluble CD163 has now been investigated in a few studies in NAFLD. Generally the levels found in NAFLD are slightly (but significantly) increased and mimics those seen in severe obesity. In obese children undergoing lifestyle intervention, we demonstrated increased sCD163 levels in children with ultrasonographic steatosis and elevated transaminases (Kazankov et al. 2015b). Furthermore, the change in sCD163 during the intervention was independently associated with the corresponding change in liver enzymes and measures of the metabolic profile such as insulin resistance (Kazankov et al. 2015b).

Two studies in morbidly obese adults set for bariatric surgery, with biopsy-proven NAFLD and NASH, showed that sCD163 increases with increasing disease severity from simple steatosis to NASH and fibrosis (Kazankov et al. 2015c; Mueller et al. 2015). This was recently verified in two populations of 157 and 174 patients with biopsy-verified NAFLD (Kazankov et al. 2016b). Significant independent associations to histological severity of NAFLD (NAS score and fibrosis scores) were demonstrated (Kazankov et al. 2016b; Mueller et al. 2015). Increased sCD163 predicted advanced fibrosis (AUROC 0.77–0.80) (Kazankov et al. 2016b). Higher levels of sCD163 were found in the systemic venous blood compared to portal circulation suggesting sCD163 release from the liver (Kazankov et al. 2015b). Following surgery, sCD163 decreased 30–40% in association with liver enzymes and improvement in insulin sensitivity irrespective of NAFLD Activity Score and fibrosis stage (Kazankov et al. 2015b) (Fig. 2). A complementary study of morbidly obese patients undergoing bariatric surgery showed 20% decreasing sCD163 levels in association with the reduction in hepatic liver fat fraction (Fjeldborg et al. 2015), and similarly sCD163 decreased (13%) in obese patients following dietary-induced weight loss (Fjeldborg et al. 2013).

In conclusion, sCD163 reflects the macrophage activation in NAFLD and is independently associated with the severity of liver disease (liver inflammation and fibrosis) and metabolic derangement in children and adults with NAFLD. This supports that macrophages play an important role in the pathogenesis of human

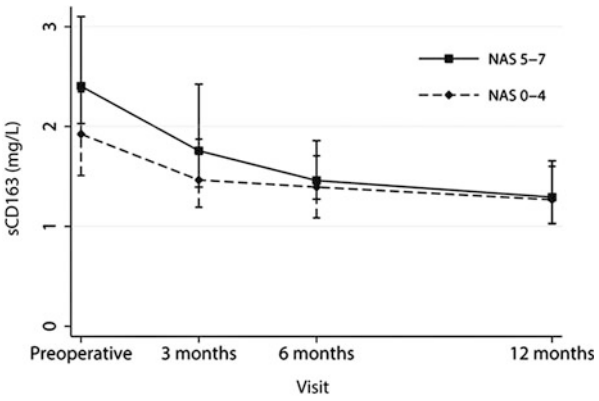

Fig. 2 Bariatric surgery normalizes sCD163 in patients with NAFLD. Levels of sCD163 in plasma following bariatric surgery in morbidly obese patients, stratified according to the NAFLD Activity Score (Reprinted from (Kazankov et al. 2015c). © 2015 Journal of Gastroenterology and Hepatology Foundation and Wiley Publishing Asia Pty Ltd, with permission)

NAFLD and NASH as suggested by experimental studies. Soluble sCD163 is a promising marker of NAFLD severity, especially liver fibrosis, and may reflect the course of disease following interventions, although further longitudinal studies, preferably with repeated liver biopsies, are needed.

sCD163 in Alcoholic Liver Disease

Alcoholic liver disease (ALD) is a heterogeneous disease entity, ranging from asymptomatic fatty liver disease, to severe AH, and progression to cirrhosis. Even though the pathogenesis of ALD continues to be only incompletely understood, activation of the Kupffer cells is important (Adachi et al. 1994, 1995; Gao and Bataller 2011). Activation of these cells may give rise to most of the hallmark clinical findings of ALD: hepatic inflammation, neutrophil recruitment, and stellate cell activation, leading to fibrosis and portal hypertension (Cavaillon 1994).

One described mechanism of Kupffer cell activation in ALD involves bacterial-derived endotoxins/LPS present in the portal blood. Such endotoxinemia due to translocation of bacteria is the result of alcohol-induced increase in the gut-blood permeability, which is found in patients with alcoholic liver injury (Fujimoto et al. 2000). LPS is recognized by the Kupffer cells via a membrane complex including the pathogen recognition receptor molecule TLR-4. LPS Binding Proteins (LBP) produced by hepatocytes then bind and present LPS to the membrane glycoprotein CD14 that in turn activates TLR-4 thus activating pro-inflammatory signaling pathways (Krasity et al. 2011). The TLR-4-mediated macrophage activation is also one described mechanism for the shedding of the CD163 receptor (Etzerodt et al. 2010).

Very limited data are available on the involvement of CD163 and sCD163 in the early stages of ALD, whereas several papers have investigated sCD163 as a biomarker in the later stages of ALD with cirrhosis and portal hypertension. In one study, we found two times increased concentrations of sCD163 in a group of hospitalized patients with chronic alcohol abuse without clinical signs of liver

cirrhosis (Møller et al. 2007). This indicates that there is a gradual increase in sCD163 in the course of ALD from fatty liver disease (30–40% increase) over severe alcohol abuse (100% increase) to cirrhosis (3–400% increase).

AH is an acute life- and health-threatening alcohol-induced hepatic inflammation with acute jaundice. The disease has a very high short- and long-term mortality, and current trends show an increasing disease incidence (Sandahl et al. 2011). There is a significant activation of macrophages in the liver of AH patients involving both pro- and anti-inflammatory phenotypes (Lee et al. 2014), and a very strong upregulation of CD163 in the Kupffer cells has been consistently described (Lee et al. 2014; Sandahl et al. 2014) (Fig. 3).

This increased expression is reflected by very high levels of sCD163 in the circulation. In a study comprising 50 patients with acute AH, the level of sCD163 was on average ten times increased (Fig. 4), almost to the levels seen in ALF (see paragraph below). At baseline the sCD163 levels significantly correlated with both the Glasgow Alcoholic Hepatitis Score (GAHS), model for end-stage liver disease MELD, and most strongly with the Child–Pugh score. There was a steady, but slow, decrease in sCD163 (24%) during the 30 days of observation. Interestingly those patients who died within the first 3 months had significantly higher levels of sCD163 at baseline than the survivors, and in multiple regression analyses GHAS and sCD163 proved to be independent predictors of mortality (Sandahl et al. 2014).

sCD163 in Hepatitis B and C

Macrophages play an important role in inflammation and fibrosis in chronic viral hepatitis B and C as recently reviewed by Boltjes et al. (2014). Early studies demonstrated elevated hepatic CD163 expression and raised serum sCD163 levels in patients with acute, compared to chronic, hepatitis B virus (HBV) infection

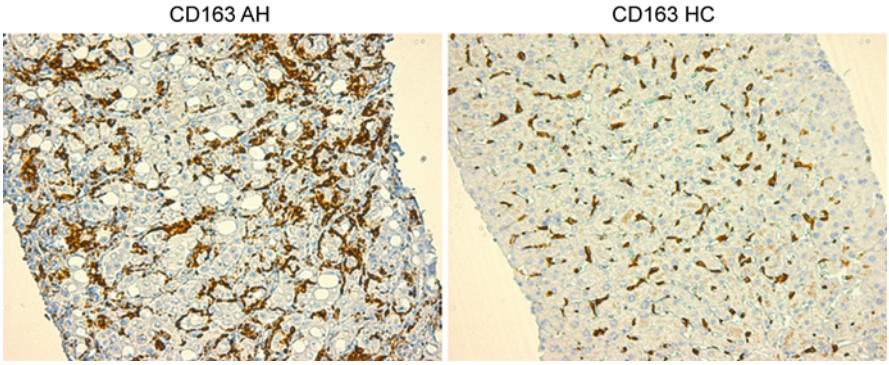

Fig. 3 Strong Kupffer cell expression of CD163 in alcoholic hepatitis. Expression of the CD163 receptor in liver biopsies from patients with alcoholic hepatitis (AH) and healthy controls (HCs) as seen by immunohistochemistry (Reprinted from (Sandahl et al. 2014). © 2014 by the American College of Gastroenterology, Rights managed by Nature Publishing Group, with permission)

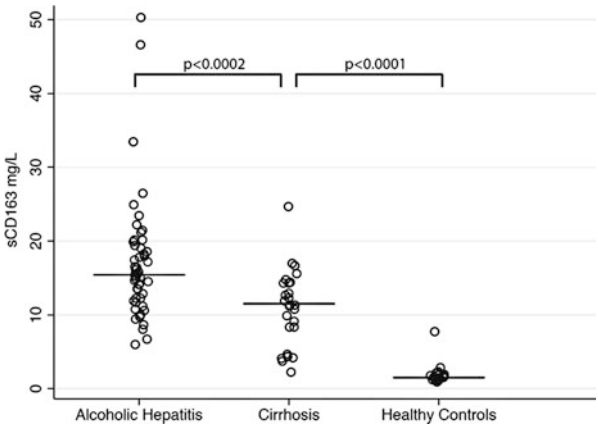

Fig. 4 Levels of sCD163 are highly increased in alcoholic hepatitis. Plasma sCD163 levels in patients with alcoholic hepatitis, patients with stable alcoholic cirrhosis, and in healthy control individuals (Reprinted from (Sandahl et al. 2014). © 2014 by the American College of Gastroenterology, Rights managed by Nature Publishing Group, with permission)

(Hiraoka et al. 2005a, b). A smaller pilot study also pointed to a possible association between sCD163 and fibrosis in hepatitis C (Andersen et al. 2014).

We have extracted data from patients with acute and chronic viral hepatitis from our recently published studies (Table 1) on patients with ALF (Møller et al. 2007), chronic hepatitis (Kazankov et al. 2014, 2015a), and cirrhosis/portal hypertension (Rode et al. 2013). This data set clearly shows sCD163 to be a marker of the disease severity, degree of inflammation, and fibrosis grade in viral hepatitis B and C, thereby supporting that macrophage activation is a central pathogenic mechanism in the disease progression.

We have demonstrated that treatment of naïve patients with chronic HBV and HCV infection have increased sCD163 levels with independent associations to histopathological scores of hepatic inflammation and fibrosis scores (Kazankov et al. 2014, 2015a). The association to histological inflammation in hepatitis B and C was recently confirmed in two independent studies that also demonstrated a significant decrease in sCD163 after successful antiviral treatment (Dultz et al. 2015, 2016).

Based on these data we developed novel simple sCD163-based fibrosis scores (FS) for significant fibrosis in chronic viral hepatitis ($\geq$F2). The CD163-HCV-FS and CD163-HBV-FS have AUROC's of 0.79 (95% CI: 0.74–0.83) and 0.71 (95% CI: 0.62–0.79), respectively (Kazankov et al. 2014). CD163-HCV-FS was superior to the apspartate transaminase (AST) to platelet ration index (APRI) and fibrosis 4 (FIB-4) models for significant fibrosis, while CD163-HBV-FS performed similar to APRI and FIB-4. Strong associations between sCD163 and APRI and FIB-4 in HCV were also recently described by Kuniholm et al. (2015).

In patients with chronic HBV and HCV infection with cirrhosis and portal hypertension, we observed higher sCD163 levels in patients with MELD $\geq$10 compared to MELD <10 (Rode et al. 2013). sCD163 levels correlated with the MELD score in patients with HCV ($n = 67$, Spearman's rho $= 0.45$, $p = 0.000$) and HBV ($n = 15$, rho $= 0.53$, $p = 0.044$). Finally, we noted increased sCD163 levels in HBV and HCV CP-B and -C patients compared to CP-A. In that study,

sCD163 levels correlated with CP-score in HCV ($n = 43$, rho = 0.62, $p = 0.000$) and HBV ($n = 12$, rho = 0.89, $p = 0.000$) (Rode et al. 2013). Thus, sCD163 represents an informative biomarker of macrophage activation in viral hepatitis reflecting disease activity and severity.

In patients with chronic HBV and HCV infection, sCD163 reflects innate immune activation that is the pathogenic basis for disease progression. The higher sCD163 in HCV vs. HBV for similar grades of fibrosis and inflammation (Table 1) may result from differences in immune cell activation pathways as reviewed by Boltjes

Table 1 Levels of sCD163 (median, IQ 25–75% range) in hepatitis B and C according to severity. Data on patients with chronic viral HBV and HCV infection were extracted from our previously published cohorts (excluding patients with other etiologies)

Severity of HBV/HCV	Number of cases (n = HBV/HCV)	HBV sCD163 (mg/L)	HCV sCD163 (mg/L)	P-value HBV vs. HCV
Scheuer fibrosis score (F0–F4)[a–c]	F0 ($n = 41/64$)	2.0 (1.6–2.6)	2.6 (1.9–3.7)	$p = 0.006$
	F1 ($n = 95/198$)	2.3 (1.8–3.0)	3.1 (2.2–4.3)	$p = 0.000$
	F2 ($n = 42/149$)	2.8 (2.0–4.3)	3.8 (2.9–5.0)	$p = 0.002$
	F3 ($n = 15/53$)	3.8 (2.5–5.8)	5.5 (3.9–6.9)	$p = 0.056$
	F4 ($n = 11/83$)	3.2 (1.8–6.8)	6.2 (4.6–8.7)	$p = 0.013$
Scheuer lobular inflammation (I0–I4)[a, b]	I0 ($n = 4/13$)	1.4 (1.1–3.3)	2.7 (1.5–3.9)	$p = 0.300$
	I1 ($n = 19/66$)	1.8 (1.6–2.6)	2.7 (1.9–3.7)	$p = 0.013$
	I2 ($n = 159/407$)	2.4 (1.9–3.4)	3.7 (2.6–5.5)	$p = 0.000$
	I3 ($n = 6/12$)	5.7 (2.4–8.1)	6.5 (4.9–8.9)	$p = 0.620$
	I4 ($n = 5/6$)	6.5 (4.3–7.7)	6.1 (5.1–11.4)	$p = 0.790$
Cirrhosis[c]	CP A ($n = 11/26$)	1.9 (1.6–2.9)	4.8 (3.6–6.4)	$p = 0.000$
	CP B-C ($n = 1/17$)	13.1	8.7 (5.5–15.2)	$p = 0.560$
	MELD <10 ($n = 12/43$)	2.4 (1.8–4.9)	5.3 (4.3–7.6)	$p = 0.003$
	MELD ≥ 10 ($n = 3/20$)	2.2 (1.6–13.1)	8.2 (6.5–14.3)	$p = 0.190$
Acute liver failure[d]	($n = 9/0$)	24.8 (12.0–30.6)		

Original reference for data extraction:
[a]Kazankov et al. 2014
[b]Kazankov et al. 2015a
[c]Rode et al. 2013
[d]Møller et al. 2007

et al. (2014), with a more aggressive inflammatory and fibrogenic milieu in HCV. Elevated sCD163 in association with MELD- and CP-score likely signify the added burden of endotoxemia, which is known to activate macrophages via the TLR-4 pathway followed by shedding of sCD163 to the circulation.

In conclusion, the breadth of current evidence suggests that sCD163 is an accurate biomarker of macrophage activation and hence liver disease activity in patients with acute and chronic viral hepatitis. Levels increase further in the context of portal hypertension and cirrhosis, and with the necroinflammation seen in acute hepatitis. Future studies should evaluate sCD163 in viral hepatitis as a marker for disease progression or resolution (following antiviral treatment). From our perspective, sCD163 holds promise as a valid surrogate marker of disease activity both for clinical management and for patient stratification in future clinical trials (Kazankov et al. 2014).

sCD163 in Cirrhosis and Portal Hypertension

Liver cirrhosis is the end stage of most chronic liver diseases. It has a poor prognosis due to the related complications of variceal bleeding, ascites and hepato-renal syndrome, and hepatic encephalopathy. The Kupffer cells and recruited macrophages play important roles in the inflammation, cell death, and fibrosis development seen in cirrhosis (Tacke and Zimmermann 2014; Seki and Schwabe 2015).

The concentration of sCD163 is highly increased in patients with liver cirrhosis (two to seven times the values in healthy) (Møller et al. 2007; Holland-Fischer et al. 2011; Grønbæk et al. 2012; Rode et al. 2013; Sandahl et al. 2014; Sprinzl et al. 2015), however, with a large variation among individual patients. This variation is related to the severity of the disease: Higher levels are seen in patients with ascites, spontaneous bacterial peritonitis, and hepato-renal syndrome as compared to stable/compensated cirrhosis (Rode et al. 2013; Waidmann et al. 2013a). The concentration is negatively associated with galactose elimination capacity (GEC) and positively with MELD (Holland-Fischer et al. 2011; Rode et al. 2013), and there is a highly significant stepwise increase with increased CP score (Grønbæk et al. 2012; Rode et al. 2013; Waidmann et al. 2013a) (Fig. 5). In cirrhosis, the association to standard liver tests is weak or absent (Holland-Fischer et al. 2011).

A hallmark in cirrhosis is the development of increase in portal pressure which is the background for cirrhosis complications and ultimately increased mortality. Interestingly, there is a strong positive correlation between sCD163 and portal hypertension, as measured by the hepatic venous pressure gradient (HVPG) in cirrhosis patients. This has been shown in two cohorts of cirrhosis patients (Holland-Fischer et al. 2011; Grønbæk et al. 2012) and further confirmed in independent studies (Waidmann et al. 2013a). AUROC for the prediction of a HVPG $>$ 10 mmHg was 0.83 yielding a sensitivity of 66% and a specificity of 94% at a sCD163 cutoff corresponding to the upper normal reference limit (Grønbæk et al. 2012).

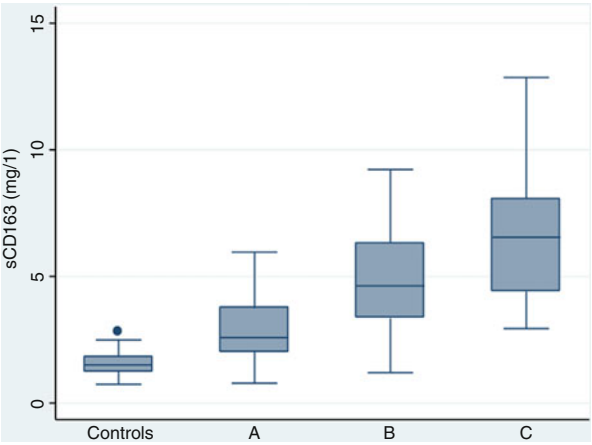

Fig. 5 Levels of sCD163 in healthy control individuals and in patients with different degrees of liver cirrhosis. Stepwise increase in levels of sCD163 with increased Child Pugh score in liver cirrhosis. A: Child-Pugh A, B: Child-Pugh B, C: Child-Pugh C (Modified from (Grønbæk et al. 2012). © 2012 Blackwell Publishing Ltd, with permission)

Kupffer cell activation plays an important role in portal hypertension, and sCD163 may be a noninvasive tool to identify cirrhosis patients with severe portal hypertension. The biological explanation for the tight association could be a direct involvement of Kupffer cells in the propagation of portal pressure by release of vasoactive substances (dynamic component) and by involvement in the formation of fibrosis (structural component) (Steib et al. 2007; Grønbæk et al. 2012). Interestingly, the sCD163 concentration did not change after installation of TIPS (Holland-Fischer et al. 2011), showing that the Kupffer cell activation is a constitutive process and that sCD163 therefore may not be suitable for monitoring a reduction in portal hypertension.

Portal hypertension leads to the formation of portocaval shunts such as esophageal varices. Supporting the relationship between sCD163 and portal pressure, a large Chinese study involving almost 1000 patients with cirrhosis, and without previous history of bleeding, showed that sCD163 was significantly elevated in the patients with varices compared to patients without (10.8 mg/L vs. 5.6 mg/L, $p = 0.015$) (Yang et al. 2013). In AUROC analysis (AUC = 0.81) a sensitivity of 80% and a specificity of 89% for the presence of varices were obtained (Yang et al. 2013).

These associations have important implications for using sCD163 as a prognostic marker in cirrhosis. In a cohort of cirrhosis patients (Waidmann et al. 2013a) it was shown that those patients with high sCD163 levels at baseline (upper 25%) had a significantly higher risk of major variceal bleeding in comparison to patients with low sCD163 serum concentration during 1–2 years of follow-up. Importantly, only sCD163 and red spots identified by gastroscopy (but not MELD or CRP) were independently associated with increased risk of variceal bleeding (Waidmann et al. 2013a). Another study showed that patients who progressed from compensated liver disease (defined as progression to CP C, MELD 15, death, or liver transplantation) had a mean 2.5 times higher sCD163 levels, and in multivariate analysis, sCD163 was a strong independent predictor of disease progression within 3 months

Table 2 Associations of sCD163 to disease status and endpoints in liver cirrhosis

- Stage of liver cirrhosis
- Hepatic decompensation
- Portal hypertension
- Presence of esophageal varies
- Risk of gastrointestinal bleeding
- Risk of disease progression
- Overall survival

(Rode et al. 2013). Similarly, the sCD163 level was a strong and independent predictor of overall survival in cirrhosis patients ($p < 0.001$) (Waidmann et al. 2013a).

In summary, sCD163 levels are associated with both pathophysiological disease status and clinical endpoints in liver cirrhosis (Table 2) and are established as a very promising biomarker to aid monitoring and treatment of these patients.

sCD163 in Acute Liver Failure

ALF is a life-threatening condition with a mortality of 40% despite intensive care treatment and liver transplantation in selected individuals (Bernsmeier et al. 2014). The high mortality results from multiorgan involvement, systemic inflammation, and secondary immune paresis with infections. A number of precipitating factors are recognized such as viral hepatitis and drug intoxication, especially acetaminophen. The diagnosis of ALF relies on clinical symptoms such as jaundice and hepatic encephalopathy combined with increased liver enzymes and coagulopathy seen in a patient without prior known liver disease.

Macrophages play a central role in the pathogenesis of both acetaminophen-induced and viral ALF (Antoniades et al. 2012; Yang et al. 2012). An accepted model emphasizes the activation of Kupffer cells and infiltrating monocytes by danger signals from necrotic hepatocytes, which results in a pro-inflammatory state with elevated cytokines. After a few days, the initial systemic inflammatory response syndrome (SIRS) is counterbalanced by a secondary resolution state where the macrophages are switched to an anti-inflammatory phenotype. The involvement of CD163+ macrophages, and the strong CD163 upregulation in ALF tissue, has been documented (Antoniades et al. 2014; Zhang et al. 2014).

Accordingly, very high levels of sCD163 are seen at admission in patients with ALF (Hiraoka et al. 2005b; Møller et al. 2007; Antoniades et al. 2013; Craig et al. 2013), with generally a tenfold increase in concentrations as compared to healthy individuals (Hiraoka et al. 2005b; Møller et al. 2007). Such high levels are otherwise only seen in the rare hemophagocytic syndrome/macrophage activation syndrome (Schaer et al. 2005), in alcoholic hepatitis (Sandahl et al. 2014), and occasionally in severe sepsis (Schaer et al. 2006). All of these conditions are

characterized by severe systemic inflammation with a high acute mortality, indicating that ALF may represent a spectrum of macrophage activation syndrome (Hiraoka et al. 2005b; Antoniades et al. 2013; Craig et al. 2013). In accordance with this, sCD163 was independently associated with liver dysfunction and mortality in a study of 1650 critically ill patients (Ingels et al. 2013). Notably, it seems that sCD163 levels in acetaminophen-induced ALF are lower compared with other etiologies (Antoniades et al. 2012; Zhang et al. 2014).

In ALF, sCD163 at admission correlates with severity of disease (MELD, organ failure scores, and international normalized ratio [INR]) (Hiraoka et al. 2005b; Møller et al. 2007; Antoniades et al. 2013), emphasizing the possible use of the biomarker for prognostic purposes. At baseline, patients with fatal outcome have significantly elevated levels compared to survivors (Møller et al. 2007; Antoniades et al. 2013; Craig et al. 2013). In one study, AUROC for separation of survivors versus nonsurvivors was 0.83 [0.69–0.96; $p = 0.0002$] (Antoniades et al. 2013); however, the time of sampling seems important. In our study (Møller et al. 2007), AUROC increased from day 1 (0.64) to day 3 of admission (0.73) and using the highest sCD163 values of day 1 and 3 showed an AUROC of 0.80 (0.66–0.95). This confirms the initial data from Hiraokoa et al. (2005b) with a progressive increase in sCD163 over time, in patients who did not survive, and a decrease in survivors (Antoniades et al. 2012).

In summary, sCD163 is highly increased in ALF, predicts fatal disease, and follows the course of disease. Apart from transplantation and treatment with N-Acethylcysteine, there are not many treatment options in ALF, and the decision whether to perform liver transplantation is difficult. Therefore biomarkers such as sCD163 that can improve existing prognostic scoring systems such as MELD and Kings College criteria are warranted. Moreover, Kupffer cells with a strong expression of CD163 may prove to be an ideal target for therapy (Possamai et al. 2014).

sCD163 in Acute-on-Chronic Liver Failure (ACLF)

Acute-on-chronic liver failure (ACLF) is a syndrome of acute deterioration in liver function in patients with preexisting chronic liver disease (typically liver cirrhosis). The precipitating event may be, e.g., hepatitis, alcohol, or infection, and the resulting inflammatory deterioration leads to organ failures with a high mortality. As in ALF, very high levels of sCD163 are seen at admission, and sCD163 correlates with severity of disease (Ye et al. 2013). A recent large study by our group, including 851 patients from the CLIF acute-on-chronic liver failure (CANONIC) trial, confirmed that sCD163 increased with increasing severity of ACLF, reaching levels seen in ALF in patients with ACLF grade III. sCD163 was independently associated with survival (both long and short term), and measurement of sCD163 alone performed at least as well as established composite prognostic scores (Grønbæk et al. 2015).

sCD163 in Hepatocellular Carcinoma (HCC)

Hepatocellular carcinoma is one of the most common cancers worldwide (Llovet et al. 2003). Surgery is potentially curative, but the patient prognosis is still poor due to high recurrence rates and the underlying liver disease. The cancer most often arises on a background of cirrhosis and chronic inflammation in the liver due to chronic viral hepatitis or ALD.

CD163+ tumor-associated macrophages (TAMs) are known to be associated with a poor prognosis in a number of different malignant diseases. This is due to the direct involvement of these cells in immune suppression, neoangiogenesis, matrix remodeling, production of growth factors, and chemotaxia (Graversen and Moestrup 2015; Sprinzl et al. 2015). At least some of the effects are mediated via STAT3 activation in both hepatocytes and CD163+ TAMs (Mano et al. 2013a).

In HCC, the number of peritumoral CD163+ macrophages are associated with the number of circulating neutrophils and are thought to play an important role for the recruitment of cytokine-producing cells to the tumor (Mano et al. 2013b; Motomura et al. 2013). Two other studies showed an increase in CD163+ TAMs (and mRNA expression) in HCC (as compared to CD68+ cells), especially in the peritumoral region (Kong et al. 2013; Yeung et al. 2015). In one study there was no correlation between the number of peritumoral CD163+ cells and survival (Kong et al. 2013), whereas another study demonstrated a significantly poor prognosis and correlation to disease aggressiveness of peritumoral CD163 infiltration (Yeung et al. 2015). An interesting case study demonstrated the presence of overactivated CD163+ cells intratumorally in a patient with spontaneous regression of a large HCC, indicating that the location and activation state (M1 vs. M2 phenotype) of the macrophages may be important for the disease course (Wang et al. 2015).

The general level of sCD163 in plasma in patients with HCC seems to reflect the inflammatory, fibrotic, and cirrhotic state of the liver in these patients, and the plasma levels therefore need to be interpreted in this context. For instance, the levels of sCD163 in HCC associate with general liver biomarkers (ALT, AST, GGT, alkaline phosphatase (ALP)) probably reflecting the background inflammation of the underlying liver disease, and the levels do not relate to the cancer as such (tumor size, microvessel invasion, or tumor nodes metastasis (TNM) stage) (Kong et al. 2013; Kazankov et al. 2016a). Similarly, sCD163 did not differ significantly between patients with HCC and cirrhotic patients without HCC (Waidmann et al. 2013b; Kazankov et al. 2016a).

One of the predominant risk factors for HCC, however, is the sustained liver inflammation (Alison et al. 2011). This may explain why the levels of sCD163 are strongly related to prognosis in patients with HCC even though sCD163 in the plasma probably does not reflect the TAMs per se. The cumulative survival of patients with low to moderate sCD163 was significantly better in two large cohorts of HCC patients compared to patients with increased sCD163 ($p = 0.005$/ $p = 0.014$) (Waidmann et al. 2013b). The sCD163 level was associated with poor survival independently of the CLIP (Cancer of the Liver Italian Program) score and the Barcelona Clinic Liver Cancer (BCLC) stage in multivariate analysis, and the

prognostic significance of sCD163 was evident in both early and advanced disease stage (Waidmann et al. 2013b). A significant relation between sCD163 levels and progression-free (but not overall) survival was also demonstrated in a recent independent study (Kazankov et al. 2016a). Interestingly yet another recent study showed a significant reduction of sCD163 in HCC patients upon treatment with the kinase-inhibitor Sorafenib which probably works by inhibiting the M2 macrophages (Sprinzl et al. 2015). These findings call for prospective monitoring of sCD163 for evaluation of the prognostic relevance during therapy in HCC.

Future Directions in Research and Clinical Use

As summarized in this review, sCD163 has now been evaluated in a large number of liver diseases and shown promising utility for diagnosis and prognosis in various clinical situations. All evidence indicates that sCD163 will also prove to be a very valuable biomarker to monitor the disease course and treatment of liver patients. Based on a low intraindividual biological variation (Møller et al. 2003), small changes in sCD163 are clinically significant.

Focus should now be on validation of published results and on translation of the findings into useful guidelines and decision limits in specific clinical situations. As a prerequisite, this will need an international standardization of commercial assays based on traceable calibrators.

Due to the increasing burden of liver diseases related to obesity and life style, there is an increasing demand for cheap, noninvasive, and reliable biomarkers that can identify individuals at risk before overt disease becomes manifest. sCD163 may potentially fill an important gap and aid in identifying individuals at high risk of developing NASH and fibrosis for focused intervention in these groups.

Potential Applications to Other Diseases or Conditions

Since sCD163 originates from macrophages in various organs, the biomarker has the potential for clinical use in a number of inflammatory, infectious, and malignant diseases characterized by macrophage involvement. Nevertheless, due to the high number of Kupffer cells and their strong CD163 expression, liver diseases presently represent the clinical field in which sCD163 has the most promising potential. The levels in inflammatory liver diseases generally greatly exceed levels in chronic inflammatory, autoimmune, and malignant diseases. Principally this separates sCD163 from more general inflammatory or acute phase markers, such as CRP and IL-6.

An increasing number of research publications has investigated the biology and clinical applicability of sCD163 in a range of diseases (Møller 2012), and the field is expanding rapidly. High levels are seldom seen outside liver disease, however, with a few exceptions: In Macrophage activation syndrome/Hemophagoctic lymphohistiocytosis (HLH), which is characterized by excessive macrophage activation,

hemophagocytosis, and cytokine storm, sCD163 reach extremely high levels. This condition is very rare, however important to recognize. Moreover, severe sepsis (including viral hemorrhagic fevers) may be accompanied by varying degrees of hemophagocytosis, and in these, cases reach high sCD163 levels. Interestingly sCD163 has, in several sepsis studies, shown a stronger association to prognosis than CRP and to better separate noninfectious systemic inflammatory response syndromes (SIRS) from sepsis. Also, patients with Gaucher disease, an hereditary deficiency of glucocerebrosidase that results in accumulation of lipid-laden macrophages in bone marrow and other organs, present with high sCD163 levels that subsequently diminish upon treatment. Much lower sCD163 levels are seen in chronic inflammatory diseases such as rheumatoid arthritis, diabetes, and HIV. The rather weak separation from normal values in these diseases hampers the clinical use of the biomarker on an individual patient basis; nevertheless the biomarker adds important information in clinical trials and in the elucidation of disease pathogenesis. The use of sCD163 in HIV is a good example. Due to the high efficiency of current HIV treatments, focus is now also on preventing long-term side effects associated with chronic inflammation and treatment, and over the last 3–4 years, sCD163 has been established as an important biomarker for evaluation of low-grade inflammation, subclinical macrophage activation, and hence atherosclerosis during medical treatment (Subramanian et al. 2012). Other applications of measuring sCD163 include the use of other biological material than serum or plasma, e.g., synovial fluid or cerebrospinal fluid. These applications allow for evaluation of the local activation of macrophages in the joints or CNS. Recently sCD163 was shown to be strongly increased in urine from patients with active vasculitis, allowing for precise diagnostics and patient management. The high urine concentrations seem to be due to release from monocyte-/macrophage-loaded crescents within the Bowman's capsule, thus offering a noninvasive supplement to renal biopsy (O'Reilly et al. 2016).

Summary Points

- Soluble CD163 (sCD163) is a circulating protein biomarker originating specifically from monocytes and macrophages, which is shed to plasma by metalloprotease action during macrophage activation.
- sCD163 can be measured precisely by commercially available ELISA kits; however, a lack of international standardization still hampers transferability of results to general clinical use.
- Due to the vast number of Kupffer cells and their strong CD163 expression, sCD163 is particularly useful in liver diseases.
- sCD163 increases from nonalcoholic fatty liver (NAFL) to steatohepatitis (NASH) and is independently associated with histological severity (NAS score and fibrosis scores).
- Very high levels that correlate with composite severity scores (such as Glasgow alcoholic hepatitis score, Model for end-stage liver disease, and Child-Pugh

score) are seen in acute alcoholic hepatitis, and the baseline levels are independent predictors of mortality.
- sCD163 is a marker of the disease severity, degree of inflammation, and fibrosis grade in viral hepatitis B and C, and recently sCD163-based fibrosis scores (FS) for significant fibrosis ($\geq$F2) have been developed.
- In cirrhosis, sCD163 associates strongly with portal hypertension, and a high sCD163 concentration at baseline is a significant risk factor for variceal bleeding, disease progression, and mortality.
- The highly increased levels of sCD163 observed in acute liver failure and acute-on-chronic liver failure follow the disease severity, and a single sCD163 result performs as well as established composite scores in prediction of fatal disease.
- sCD163 may be useful in several other inflammatory diseases, and its macrophage specificity generates clinical information that is not obtained by traditional inflammatory or acute phase markers.

References

Adachi Y, Bradford BU, Gao W, Bojes HK, Thurman RG. Inactivation of Kupffer cells prevents early alcohol-induced liver injury. Hepatology. 1994;20(2):453–60.

Adachi Y, Moore LE, Bradford BU, Gao W, Thurman RG. Antibiotics prevent liver injury in rats following long-term exposure to ethanol. Gastroenterology. 1995;108(1):218–24.

Alison MR, Nicholson LJ, Lin WR. Chronic inflammation and hepatocellular carcinoma. Recent Results Cancer Res. 2011;185:135–48.

Andersen CB, Moestrup SK. How calcium makes endocytic receptors attractive. Trends Biochem Sci. 2014;39(2):82–90.

Andersen ES, Rødgaard-Hansen S, Moessner B, Christensen PB, Møller HJ, Weis N. Macrophage-related serum biomarkers soluble CD163 (sCD163) and soluble mannose receptor (sMR) to differentiate mild liver fibrosis from cirrhosis in patients with chronic hepatitis C: a pilot study. Eur J Clin Microbiol Infect Dis. 2014;33(1):117–22.

Antoniades CG, Quaglia A, Taams LS, Mitry RR, Hussain M, Abeles R, Possamai LA, Bruce M, McPhail M, Starling C, Wagner B, Barnardo A, Pomplun S, Auzinger G, Bernal W, Heaton N, Vergani D, Thursz MR, Wendon J. Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans. Hepatology. 2012;56(2):735–46.

Antoniades C, Triantafyllou E, Gadhok R, Abeles R, Quaglia A, Khamri W, Tidswell R, McPhail M, Possamai L, Bernal W, Heneghan M, Ma Y, Auzinger G, Heaton N, Thursz M, Wendon J. 1013 Cd163 Is a mechanistic biomarker in acute liver failure reflecting a macrophage activation like syndrome. J Hepatol. 2013;58:S417.

Antoniades CG, Khamri W, Abeles RD, Taams LS, Triantafyllou E, Possamai LA, Bernsmeier C, Mitry RR, O'Brien A, Gilroy D, Goldin R, Heneghan M, Heaton N, Jassem W, Bernal W, Vergani D, Ma Y, Quaglia A, Wendon J, Thursz M. Secretory leukocyte protease inhibitor: a pivotal mediator of anti-inflammatory responses in acetaminophen-induced acute liver failure. Hepatology. 2014;59(4):1564–76.

Bauer S, Weiss TS, Wiest R, Schacherer D, Hellerbrand C, Farkas S, Scherer MN, Ritter M, Schmitz G, Schaffler A, Buechler C. Soluble CD163 is not increased in visceral fat and steatotic liver and is even suppressed by free fatty acids in vitro. Exp Mol Pathol. 2011;91(3):733–9.

Bernsmeier C, Antoniades CG, Wendon J. What's new in acute liver failure? Intensive Care Med. 2014;40(10):1545–8.

Bohm T, Berger H, Nejabat M, Riegler T, Kellner F, Kuttke M, Sagmeister S, Bazanella M, Stolze K, Daryabeigi A, Bintner N, Murkovic M, Wagner KH, Schulte-Hermann R,

Rohr-Udilova N, Huber W, Grasl-Kraupp B. Food-derived peroxidized fatty acids may trigger hepatic inflammation: a novel hypothesis to explain steatohepatitis. J Hepatol. 2013;59(3):563–70.
Boltjes A, Movita D, Boonstra A, Woltman AM. The role of Kupffer cells in hepatitis B and hepatitis C virus infections. J Hepatol. 2014;61:660.
Bover LC, Cardo-Vila M, Kuniyasu A, Sun J, Rangel R, Takeya M, Aggarwal BB, Arap W, Pasqualini R. A previously unrecognized protein-protein interaction between TWEAK and CD163: potential biological implications. J Immunol. 2007;178(12):8183–94.
Cavaillon JM. Cytokines and macrophages. Biomed Pharmacother. 1994;48(10):445–53.
Craig DG, Lee P, Pryde EA, Hayes PC, Simpson KJ. Serum neopterin and soluble CD163 as markers of macrophage activation in paracetamol (acetaminophen)-induced human acute liver injury. Aliment Pharmacol Ther. 2013;38(11–12):1395–404.
De Vito R, Alisi A, Masotti A, Ceccarelli S, Panera N, Citti A, Salata M, Valenti L, Feldstein AE, Nobili V. Markers of activated inflammatory cells correlate with severity of liver damage in children with nonalcoholic fatty liver disease. Int J Mol Med. 2012;30(1):49–56.
Droste A, Sorg C, Hogger P. Shedding of CD163, a novel regulatory mechanism for a member of the scavenger receptor cysteine-rich family. Biochem Biophys Res Commun. 1999;256(1): 110–3.
Dultz G, Gerber L, Farnik H, Berger A, Vermehren J, Pleli T, Zeuzem S, Piiper A, Kronenberger B, Waidmann O. Soluble CD163 is an indicator of liver inflammation and fibrosis in patients chronically infected with the hepatitis B virus. J Viral Hepat. 2015;22(4):427–32.
Dultz G, Gerber L, Zeuzem S, Sarrazin C, Waidmann O. The macrophage activation marker CD163 is associated with IL28B genotype and hepatic inflammation in chronic hepatitis C virus infected patients. J Viral Hepat. 2016;23(4):267–73.
Eckert C, Klein N, Kornek M, Lukacs-Kornek V. The complex myeloid network of the liver with diverse functional capacity at steady state and in inflammation. Front Immunol. 2015;6:179.
Etzerodt A, Moestrup SK. CD163 and inflammation: biological, diagnostic, and therapeutic aspects. Antioxid Redox Signal. 2013;18(17):2352–63.
Etzerodt A, Maniecki MB, Møller K, Møller HJ, Moestrup SK. Tumor necrosis factor alpha-converting enzyme (TACE/ADAM17) mediates ectodomain shedding of the scavenger receptor CD163. J Leukoc Biol. 2010;88(6):1201–5.
Fabriek BO, van Bruggen R, Deng DM, Ligtenberg AJ, Nazmi K, Schornagel K, Vloet RP, Dijkstra CD, van den Berg TK. The macrophage scavenger receptor CD163 functions as an innate immune sensor for bacteria. Blood. 2009;113(4):887–92.
Fjeldborg K, Christiansen T, Bennetzen M, Møller HJ, Pedersen SB, Richelsen B. The macrophage-specific serum marker, soluble CD163, is increased in obesity and reduced after dietary-induced weight loss. Obesity (Silver Spring). 2013;21(12):2437–43.
Fjeldborg K, Pedersen SB, Møller HJ, Rask P, Danielsen AV, Stodkilde-Jorgensen H, Richelsen B. Intrahepatic fat content correlates with soluble CD163 in relation to weight loss induced by Roux-en-Y gastric bypass. Obesity (Silver Spring). 2015;23(1):154–61.
Fujimoto M, Uemura M, Nakatani Y, Tsujita S, Hoppo K, Tamagawa T, Kitano H, Kikukawa M, Ann T, Ishii Y, Kojima H, Sakurai S, Tanaka R, Namisaki T, Noguchi R, Higashino T, Kikuchi E, Nishimura K, Takaya A, Fukui H. Plasma endotoxin and serum cytokine levels in patients with alcoholic hepatitis: relation to severity of liver disturbance. Alcohol Clin Exp Res. 2000;24(4 Suppl):48S–54.
Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. Gastroenterology. 2011;141(5):1572–85.
Graversen JH, Moestrup SK. Drug trafficking into macrophages via the endocytotic receptor CD163. Membranes (Basel). 2015;5(2):228–52.
Grønbæk H, Sandahl TD, Mortensen C, Vilstrup H, Møller HJ, Møller S. Soluble CD163, a marker of Kupffer cell activation, is related to portal hypertension in patients with liver cirrhosis. Aliment Pharmacol Ther. 2012;36(2):173–80.
Grønbæk H, Rødgaard-Hansen S, Aagaard NK, Arroyo V, Moestrup SK, Garcia E, Sola E, Domenicali M, Piano S, Vilstrup H, Møller HJ, CANONIC Study Investigators of the

EASL-CLIF Consortium. Macrophage activation markers predict mortality in patients with liver cirrhosis without or with acute-on-chronic liver failure (ACLF). J Hepatol. 2015;64:813.

Hintz KA, Rassias AJ, Wardwell K, Moss ML, Morganelli PM, Pioli PA, Givan AL, Wallace PK, Yeager MP, Guyre PM. Endotoxin induces rapid metalloproteinase-mediated shedding followed by up-regulation of the monocyte hemoglobin scavenger receptor CD163. J Leukoc Biol. 2002;72(4):711–7.

Hiraoka A, Horiike N, Akbar SM, Michitaka K, Matsuyama T, Onji M. Expression of CD163 in the liver of patients with viral hepatitis. Pathol Res Pract. 2005a;201(5):379–84.

Hiraoka A, Horiike N, Akbar SM, Michitaka K, Matsuyama T, Onji M. Soluble CD163 in patients with liver diseases: very high levels of soluble CD163 in patients with fulminant hepatic failure. J Gastroenterol. 2005b;40(1):52–6.

Holland-Fischer P, Grønbæk H, Sandahl TD, Moestrup SK, Riggio O, Ridola L, Aagaard NK, Møller HJ, Vilstrup H. Kupffer cells are activated in cirrhotic portal hypertension and not normalised by TIPS. Gut. 2011;60(10):1389–93.

Ilan Y. Leaky gut and the liver: a role for bacterial translocation in nonalcoholic steatohepatitis. World J Gastroenterol. 2012;18(21):2609–18.

Ingels C, Møller HJ, Hansen TK, Wouters PJ, Vanhorebeek I, Van den Berghe G. Circulating levels of the shed scavenger receptor sCD163 and association with outcome of critically ill patients. J Clin Immunol. 2013;33(3):619–29.

Kazankov K, Barrera F, Møller HJ, Bibby BM, Vilstrup H, George J, Grønbæk H. Soluble CD163, a macrophage activation marker, is independently associated with fibrosis in patients with chronic viral hepatitis B and C. Hepatology. 2014;60(2):521–30.

Kazankov K, Møller HJ, Bibby BM, Vilstrup H, George J, Grønbæk H. Reply: to. Hepatology. 2015a;61(2):735–6.

Kazankov K, Møller HJ, Lange A, Birkebaek NH, Holland-Fischer P, Solvig J, Horlyck A, Kristensen K, Rittig S, Handberg A, Vilstrup H, Grønbæk H. The macrophage activation marker sCD163 is associated with changes in NAFLD and metabolic profile during lifestyle intervention in obese children. Pediatr Obes. 2015b;10(3):226–33.

Kazankov K, Tordjman J, Møller HJ, Vilstrup H, Poitou C, Bedossa P, Bouillot JL, Clement K, Grønbæk H. Macrophage activation marker soluble CD163 and non-alcoholic fatty liver disease in morbidly obese patients undergoing bariatric surgery. J Gastroenterol Hepatol. 2015c; 30(8):1293–300.

Kazankov K, Rode A, Simonsen K, Villadsen GE, Nicoll A, Møller HJ, Lim L, Angus P, Kronborg I, Arachchi N, Gorelik A, Liew D, Vilstrup H, Frystyk J, Grønbæk H. Macrophage activation marker soluble CD163 may predict disease progression in hepatocellular carcinoma. Scand J Clin Lab Invest. 2016a;76(1):64–73.

Kazankov K, Barrera F, Møller HJ, Rosso C, Bugianesi E, David E, Ibrahim Kamal Jouness R, Esmaili S, Eslam M, McLeod D, Bibby BM, Vilstrup H, George J, Grønbaek H. The macrophage activation marker sCD163 is associated with morphological disease stages in patients with non-alcoholic fatty liver disease. Liver Int. 2016b. doi:10.1111/liv.13150. [Epub ahead of print].

Kneidl J, Loffler B, Erat MC, Kalinka J, Peters G, Roth J, Barczyk K. Soluble CD163 promotes recognition, phagocytosis and killing of *Staphylococcus aureus* via binding of specific fibronectin peptides. Cell Microbiol. 2012;14(6):914–36.

Kodama Y, Kisseleva T, Iwaisako K, Miura K, Taura K, De Minicis S, Osterreicher CH, Schnabl B, Seki E, Brenner DA. c-Jun N-terminal kinase-1 from hematopoietic cells mediates progression from hepatic steatosis to steatohepatitis and fibrosis in mice. Gastroenterology. 2009;137(4): 1467–77 e1465.

Kong LQ, Zhu XD, Xu HX, Zhang JB, Lu L, Wang WQ, Zhang QB, Wu WZ, Wang L, Fan J, Tang ZY, Sun HC. The clinical significance of the CD163+ and CD68+ macrophages in patients with hepatocellular carcinoma. PLoS One. 2013;8(3):e59771.

Kracmerova J, Rossmeislova L, Kovacova Z, Klimcakova E, Polak J, Tencerova M, Malisova L, Stich V, Langin D, Siklova M. Soluble CD163 is associated with CD163 mRNA expression in

adipose tissue and with insulin sensitivity in steady-state condition but not in response to calorie restriction. J Clin Endocrinol Metab. 2014;99(3):E528–35.
Krasity BC, Troll JV, Weiss JP, McFall-Ngai MJ. LBP/BPI proteins and their relatives: conservation over evolution and roles in mutualism. Biochem Soc Trans. 2011;39(4):1039–44.
Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, Moestrup SK. Identification of the haemoglobin scavenger receptor. Nature. 2001;409(6817):198–201.
Kuniholm MH, Hanna DB, Landay AL, Kaplan RC, Ley K. Soluble CD163 is associated with noninvasive measures of liver fibrosis in hepatitis C virus- and hepatitis C virus/human immunodeficiency virus-infected women. Hepatology. 2015;61(2):734–5.
Law SK, Micklem KJ, Shaw JM, Zhang XP, Dong Y, Willis AC, Mason DY. A new macrophage differentiation antigen which is a member of the scavenger receptor superfamily. Eur J Immunol. 1993;23(9):2320–5.
Lee J, French B, Morgan T, French SW. The liver is populated by a broad spectrum of markers for macrophages. In alcoholic hepatitis the macrophages are M1 and M2. Exp Mol Pathol. 2014;96(1):118–25.
Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet. 2003;362(9399):1907–17.
Maniecki MB, Etzerodt A, Moestrup SK, Møller HJ, Graversen JH. Comparative assessment of the recognition of domain-specific CD163 monoclonal antibodies in human monocytes explains wide discrepancy in reported levels of cellular surface CD163 expression. Immunobiology. 2011;216(8):882–90.
Mano Y, Aishima S, Fujita N, Tanaka Y, Kubo Y, Motomura T, Taketomi A, Shirabe K, Maehara Y, Oda Y. Tumor-associated macrophage promotes tumor progression via STAT3 signaling in hepatocellular carcinoma. Pathobiology. 2013a;80(3):146–54.
Mano Y, Shirabe K, Yamashita Y, Harimoto N, Tsujita E, Takeishi K, Aishima S, Ikegami T, Yoshizumi T, Yamanaka T, Maehara Y. Preoperative neutrophil-to-lymphocyte ratio is a predictor of survival after hepatectomy for hepatocellular carcinoma: a retrospective analysis. Ann Surg. 2013b;258(2):301–5.
Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep. 2014;6:13.
Miura K, Yang L, van Rooijen N, Brenner DA, Ohnishi H, Seki E. Toll-like receptor 2 and palmitic acid cooperatively contribute to the development of nonalcoholic steatohepatitis through inflammasome activation in mice. Hepatology. 2013;57(2):577–89.
Møller HJ. Soluble CD163. Scand J Clin Lab Invest. 2012;72(1):1–13.
Møller HJ, Hald K, Moestrup SK. Characterization of an enzyme-linked immunosorbent assay for soluble CD163. Scand J Clin Lab Invest. 2002a;62(4):293–9.
Møller HJ, Peterslund NA, Graversen JH, Moestrup SK. Identification of the hemoglobin scavenger receptor/CD163 as a natural soluble protein in plasma. Blood. 2002b;99(1):378–80.
Møller HJ, Petersen PH, Rejnmark L, Moestrup SK. Biological variation of soluble CD163. Scand J Clin Lab Invest. 2003;63(1):15–21.
Møller HJ, Grønbæk H, Schiodt FV, Holland-Fischer P, Schilsky M, Munoz S, Hassanein T, Lee WM, U. S. A. L. F. S. Group. Soluble CD163 from activated macrophages predicts mortality in acute liver failure. J Hepatol. 2007;47(5):671–6.
Møller HJ, Nielsen MJ, Maniecki MB, Madsen M, Moestrup SK. Soluble macrophage-derived CD163: a homogenous ectodomain protein with a dissociable haptoglobin-hemoglobin binding. Immunobiology. 2010;215(5):406–12.
Møller HJ, Frikke-Schmidt R, Moestrup SK, Nordestgaard BG, Tybjaerg-Hansen A. Serum soluble CD163 predicts risk of type 2 diabetes in the general population. Clin Chem. 2011;57(2):291–7.
Møller HJ, Frikke-Schmidt R, Moestrup SK, Nordestgaard BG, Tybjærg-Hansen A. 1417 the macrophage-derived serum biomarker soluble Cd163 independently predicts liver cirrhosis in the general population. J Hepatol. 2012;56:S558.
Motomura T, Shirabe K, Mano Y, Muto J, Toshima T, Umemoto Y, Fukuhara T, Uchiyama H, Ikegami T, Yoshizumi T, Soejima Y, Maehara Y. Neutrophil-lymphocyte ratio reflects

hepatocellular carcinoma recurrence after liver transplantation via inflammatory microenvironment. J Hepatol. 2013;58(1):58–64.

Mueller JL, Feeney ER, Zheng H, Misdraji J, Kruger AJ, Alatrakchi N, King LY, Gelrud L, Corey KE, Chung RT. Circulating soluble CD163 is associated with steatohepatitis and advanced fibrosis in nonalcoholic fatty liver disease. Clin Transl Gastroenterol. 2015;6:e114.

O'Reilly VP, Wong L, Kennedy C, Elliot LA, O'Meachair S, Coughlan AM, O'Brien EC, Ryan MM, Sandoval D, Connolly E, Dekkema GJ, Lau J, Abdulahad WH, Sanders JF, Heeringa P, Buckley C, O'Brien C, Finn S, Cohen CD, Lindemeyer MT, Hickey FB, O'Hara PV, Feighery C, Moran SM, Mellotte G, Clarkson MR, Dorman AJ, Murray PT, Little MA. Urinary soluble CD163 in active renal vasculitis. J Am Soc Nephrol. 2016;27:2906.

Parkner T, Sørensen LP, Nielsen AR, Fischer CP, Bibby BM, Nielsen S, Pedersen BK, Møller HJ. Soluble CD163: a biomarker linking macrophages and insulin resistance. Diabetologia. 2012;55(6):1856–62.

Possamai LA, Thursz MR, Wendon JA, Antoniades CG. Modulation of monocyte/macrophage function: a therapeutic strategy in the treatment of acute liver failure. J Hepatol. 2014;61(2): 439–45.

Pulford K, Micklem K, McCarthy S, Cordell J, Jones M, Mason DY. A monocyte/macrophage antigen recognized by the four antibodies GHI/61, Ber-MAC3, Ki-M8 and SM4. Immunology. 1992;75(4):588–95.

Rode A, Nicoll A, Møller HJ, Lim L, Angus PW, Kronborg I, Arachchi N, Gorelik A, Liew D, Kazankov K, Vilstrup H, Grønbæk H. Hepatic macrophage activation predicts clinical decompensation in chronic liver disease. Gut. 2013;62(8):1231–2.

Sanchez-Torres C, Gomez-Puertas P, Gomez-del-Moral M, Alonso F, Escribano JM, Ezquerra A, Dominguez J. Expression of porcine CD163 on monocytes/macrophages correlates with permissiveness to African swine fever infection. Arch Virol. 2003;148(12):2307–23.

Sandahl TD, Jepsen P, Thomsen KL, Vilstrup H. Incidence and mortality of alcoholic hepatitis in Denmark 1999–2008: a nationwide population based cohort study. J Hepatol. 2011;54(4): 760–4.

Sandahl TD, Grønbæk H, Møller HJ, Stoy S, Thomsen KL, Dige AK, Agnholt J, Hamilton-Dutoit S, Thiel S, Vilstrup H. Hepatic macrophage activation and the LPS pathway in patients with alcoholic hepatitis: a prospective cohort study. Am J Gastroenterol. 2014;109(11):1749–56.

Schaer DJ, Schleiffenbaum B, Kurrer M, Imhof A, Bachli E, Fehr J, Møller HJ, Moestrup SK, Schaffner A. Soluble hemoglobin-haptoglobin scavenger receptor CD163 as a lineage-specific marker in the reactive hemophagocytic syndrome. Eur J Haematol. 2005;74(1):6–10.

Schaer DJ, Schaer CA, Schoedon G, Imhof A, Kurrer MO. Hemophagocytic macrophages constitute a major compartment of heme oxygenase expression in sepsis. Eur J Haematol. 2006;77(5):432–6.

Seki E, Schwabe RF. Hepatic inflammation and fibrosis: functional links and key pathways. Hepatology. 2015;61(3):1066–79.

Sprinzl MF, Puschnik A, Schlitter AM, Schad A, Ackermann K, Esposito I, Lang H, Galle PR, Weinmann A, Heikenwalder M, Protzer U. Sorafenib inhibits macrophage-induced growth of hepatoma cells by interference with insulin-like growth factor-1 secretion. J Hepatol. 2015; 62(4):863–70.

Steib CJ, Gerbes AL, Bystron M, Op den Winkel M, Hartl J, Roggel F, Prufer T, Goke B, Bilzer M. Kupffer cell activation in normal and fibrotic livers increases portal pressure via thromboxane A(2). J Hepatol. 2007;47(2):228–38.

Stienstra R, Saudale F, Duval C, Keshtkar S, Groener JE, van Rooijen N, Staels B, Kersten S, Muller M. Kupffer cells promote hepatic steatosis via interleukin-1beta-dependent suppression of peroxisome proliferator-activated receptor alpha activity. Hepatology. 2010;51(2): 511–22.

Subramanian S, Tawakol A, Burdo TH, Abbara S, Wei J, Vijayakumar J, Corsini E, Abdelbaky A, Zanni MV, Hoffmann U, Williams KC, Lo J, Grinspoon SK. Arterial inflammation in patients with HIV. JAMA. 2012;308(4):379–86.

Sulahian TH, Hintz KA, Wardwell K, Guyre PM. Development of an ELISA to measure soluble CD163 in biological fluids. J Immunol Methods. 2001;252(1–2):25–31.

Tacke F, Zimmermann HW. Macrophage heterogeneity in liver injury and fibrosis. J Hepatol. 2014;60(5):1090–6.

Tomita K, Tamiya G, Ando S, Ohsumi K, Chiyo T, Mizutani A, Kitamura N, Toda K, Kaneko T, Horie Y, Han JY, Kato S, Shimoda M, Oike Y, Tomizawa M, Makino S, Ohkura T, Saito H, Kumagai N, Nagata H, Ishii H, Hibi T. Tumour necrosis factor alpha signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. Gut. 2006;55(3):415–24.

Van Gorp H, Van Breedam W, Delputte PL, Nauwynck HJ. Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. J Gen Virol. 2008; 89(Pt 12):2943–53.

Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther. 2011;34(3):274–85.

Waidmann O, Brunner F, Herrmann E, Zeuzem S, Piiper A, Kronenberger B. Macrophage activation is a prognostic parameter for variceal bleeding and overall survival in patients with liver cirrhosis. J Hepatol. 2013a;58(5):956–61.

Waidmann O, Koberle V, Bettinger D, Trojan J, Zeuzem S, Schultheiss M, Kronenberger B, Piiper A. Diagnostic and prognostic significance of cell death and macrophage activation markers in patients with hepatocellular carcinoma. J Hepatol. 2013b;59(4):769–79.

Wang Z, Ke ZF, Lu XF, Luo CJ, Liu YD, Lin ZW, Wang LT. The clue of a possible etiology about spontaneous regression of hepatocellular carcinoma: a perspective on pathology. OncoTargets Ther. 2015;8:395–400.

Yang Q, Shi Y, He J, Chen Z. The evolving story of macrophages in acute liver failure. Immunol Lett. 2012;147(1–2):1–9.

Yang YY, Hou MC, Lin MW, Chen PH, Liao WC, Chu CJ, Lin HC. Combined platelet count with sCD163 and genetic variants optimizes esophageal varices prediction in cirrhotic patients. J Gastroenterol Hepatol. 2013;28(1):112–21.

Ye D, Li FY, Lam KS, Li H, Jia W, Wang Y, Man K, Lo CM, Li X, Xu A. Toll-like receptor-4 mediates obesity-induced non-alcoholic steatohepatitis through activation of X-box binding protein-1 in mice. Gut. 2012;61(7):1058–67.

Ye H, Wang LY, Zhao J, Wang K. Increased CD163 expression is associated with acute-on-chronic hepatitis B liver failure. World J Gastroenterol. 2013;19(18):2818–25.

Yeung OW, Lo CM, Ling CC, Qi X, Geng W, Li CX, Ng KT, Forbes SJ, Guan XY, Poon RT, Fan ST, Man K. Alternatively activated (M2) macrophages promote tumour growth and invasiveness in hepatocellular carcinoma. J Hepatol. 2015;62(3):607–16.

Zanni MV, Burdo TH, Makimura H, Williams KC, Grinspoon SK. Relationship between monocyte/macrophage activation marker soluble CD163 and insulin resistance in obese and normal-weight subjects. Clin Endocrinol (Oxf). 2012;77(3):385–90.

Zhang M, Ye Y, Wang F, Zhu J, Zhao Q, Zheng Y, Gu Y, Xie C, Huang Z, Tai Q, Chong Y, Gao Z. Liver myofibroblasts up-regulate monocyte CD163 expression via PGE2 during hepatitis B induced liver failure. J Transl Med. 2014;12:60.

CD133 and EpCAM as Biomarkers in Liver Diseases

16

Anthony W. H. Chan and Ka-Fai To

Contents

Abstract

Liver cancer is the sixth commonest malignancy and the second leading cause of cancer-associated death worldwide in 2012. Tumor recurrence after curative treatment and resistance to chemo-/radiotherapy are two important factors contributing to poor prognosis of liver cancer. Cancer stem cell (CSC) is postulated to be responsible for tumor initiation, recurrence, dissemination, and therapeutic

A.W.H. Chan • K.-F. To (✉)
Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Shatin, NT, Hong Kong
e-mail: awh_chan@cuhk.edu.hk; kfto@cuhk.edu.hk

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_12

resistance. CD133 and EpCAM are putative stemness markers of stem/progenitor cells and CSC in the liver. CD133 has unclear normal physiological function but is suggested to be in intercellular communication. Several aberrant signaling pathways are participated in CD133-positive hepatic cancers: Hedgehog, mTOR, IL-8/CXCL1, BMP/Smad, MAPK/Erk, and hypoxia-inducible factor 1-α/Notch signaling pathways. EpCAM has complex physiological functions including cell-cell adhesion, regulation of proliferation, differentiation, migration, and cell survival. Wnt/β-catenin signaling pathway is the major pathway involved in EpCAM-positive hepatic cancers. CD133 and EpCAM have several significant clinical implications in diagnosis, prognosis, and therapy of human liver cancers. Recently defined histological types of liver cancer, namely, combined hepatocellular-cholangiocarcinoma with stem cell features and hepatocellular carcinoma expressing stemness marker, require CD133, EpCAM, and/or other stemness markers to establish the diagnosis. CD133 and EpCAM are also important prognostic biomarkers. CD133 is expressed in 22.0 ± 12.1% of hepatocellular carcinoma and associated with higher histological grade, more advanced tumor stage, and worse overall and disease-free survival. EpCAM is expressed in 32.4 ± 12.8% of hepatocellular carcinoma and associated with younger age, higher histological grade, vascular invasion, more advanced tumor stage, and worse overall and disease-free survival. The prognostic roles of CD133 and EpCAM in intrahepatic cholangiocarcinoma and hepatoblastoma are less well defined. Although targeted therapies against CD133 and EpCAM against liver cancers are still in preclinical stages, they are promising new therapeutic research areas to combat the key player, cancer stem cell, responsible for tumor recurrence and therapeutic resistance liver cancers.

Keywords

CD133 • Cholangiocarcinoma • EpCAM • Hepatocellular carcinoma • Hepatoblastoma • Liver cancer • Progenitor cell • Stem cell • Stemness marker

List of Abbreviations

AFP	Alpha-fetoprotein
AJCC	American Joint Committee on Cancer
BCLC	Barcelona clinic liver cancer
BMP	Bone morphogenetic protein
CAM	Cell adhesion molecule
CK19	Cytokeratin 19
CSC	Cancer stem cell
CXCL1	Chemokine C-X-C motif ligand 1
DNMT	DNA methyltransferase
EpCAM	Epithelial cell adhesion molecule
EpEx	EpCAM extracellular domain
EpICD	EpCAM intracytoplasmic domain

EZH2	Enhancer of zeste homologue 2
HCC	Hepatocellular carcinoma
HCC-CC	Combined hepatocellular-cholangiocarcinoma
HH	Hedgehog
HSPC	Hepatic stem/progenitor cell
IHCC	Intrahepatic cholangiocarcinoma
IL-8	Interleukin-8
Line-1	Long interspersed nucleotide element-1
MAPK	Mitogen-activated protein kinase
miR	microRNA
mTOR	Mechanistic target of rapamycin
NCAM	Neural cell adhesion molecule
TGF	Transforming growth factor
TP53INP1	Tumor protein 53-induced nuclear protein 1
TROP1	Trophoblast cell-surface antigen 1

Key Facts of CD133

- CD133 is a putative marker of hepatic stem/progenitor cells and hepatic cancer stem cells.
- The function of CD133 remains unclear but it is probably involved in intercellular communication.
- Several aberrant signaling pathways could be present in CD133-positive hepatic cancers: Hedgehog, mTOR, IL-8/CXCL1, BMP/Smad, MAPK/Erk, and hypoxia-inducible factor 1-α/Notch signaling pathways.
- CD133 is expressed in 0–40.0% (mean 22.0 ± 12.1%) of hepatocellular carcinoma and associated with higher histological grade, more advanced tumor stage, and worse overall and disease-free survival.
- The prognostic role of CD133 in intrahepatic cholangiocarcinoma and hepatoblastoma is less well defined.
- Anti-CD133 therapy against HCC is still in preclinical setting.
- CD133 is a potential negative surrogate marker predicting clinical response to sorafenib.

Key Facts of EpCAM

- EpCAM is a putative marker of hepatic stem/progenitor cells and hepatic cancer stem cells.
- The function of EpCAM is complex and includes cell-cell adhesion, regulation of proliferation, differentiation, migration, and cell survival.
- Wnt/β-catenin signaling pathway is the major pathway involved in EpCAM-positive hepatic cancers.

- EpCAM is expressed in 15.9–48.7% (mean 32.4 ± 12.8%) of hepatocellular carcinoma and associated with younger age, higher histological grade, vascular invasion, more advanced tumor stage, and worse overall and disease-free survival.
- EpCAM expression in tumorous stroma instead of malignant epithelial cells has prognostic significance in intrahepatic cholangiocarcinoma.
- The prognostic role of EpCAM in hepatoblastoma is not well defined.
- Anti-EpCAM therapy against HCC is still in preclinical setting.

Definition of Words and Terms

Cancer stem cells	Malignant counterpart of normal stem cells and defined as a small subpopulation of tumor cells with features resembling normal stem cells including unlimited abilities of self-renewal, replication, and pluripotent differentiation.
Combined hepatocellular-cholangiocarcinoma with stem cell features	A variant of combined hepatocellular-cholangiocarcinoma possesses small primitive cells exhibiting stemness markers.
Ductular reaction	It is composed of proliferation of bile ductules associated with inflammatory infiltrate in the periportal region. It is regarded as an evidence of the activation of hepatic stem/progenitor cells and commonly found in various acute and chronic hepatic diseases.
Hepatocellular carcinoma expressing stemness markers	A histological variant of hepatocellular carcinoma has morphological features of classical hepatocellular carcinoma together with expression of stemness markers in more than 5% of tumor cells.
Progenitor cells	Also known as transit-amplifying cells. Intermediate cells, which are differentiated from stem cells, undergo limited number of replication and finally differentiate into specialized cells.
*S*cirrhous hepatocellular carcinoma	An uncommon histological variant accounting for 5% of all hepatocellular carcinoma. It is characterized by trabeculae, nests, and sheets of malignant hepatocytes embedded in marked fibrotic stroma (30–50% of tumor area).

Self-renewal	Production of one or two daughter cells with same abilities of replication and differentiation as the parental cells through symmetric or asymmetric self-renewal cell division.
Specialized cells	Terminally differentiated cells, which are differentiated from progenitor cell, do not divide (or rarely divide) or differentiate anymore.
Stem cells	Undifferentiated primitive cells have unlimited abilities of self-renewal, replication, and pluripotent differentiation.
Stemness marker	A marker could highlight those potential stem cells.

Introduction

Liver cancer is the sixth commonest malignancy and the second leading cause of cancer-associated death worldwide in 2012 (Ferlay et al. 2012). Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (IHCC) are two most frequent liver cancers in adults, whereas hepatoblastoma is the commonest liver cancer in children (Nakashima et al. 2010; Theise et al. 2010a; Zimmermann and Saxena 2010). The clinical outcomes of HCC and IHCC are dismal with mortality-to-incidence ratio over 90% and 5-year overall survival rate below 10%. Tumor recurrence after curative treatment and resistance to chemo-/radiotherapy are two important factors contributing to poor prognosis of HCC and IHCC. To overcome these challenges, better understanding of the underlying mechanisms associated with relapse and resistance to therapy is crucial. Cancer stem cell (CSC) may be one of the promising solutions as it is postulated to be responsible for tumor initiation, recurrence, dissemination, and therapeutic resistance (Clarke et al. 2006; Nguyen et al. 2012; Ajani et al. 2015). After the first discovery of CSC in solid cancer in 2003, researches on CSCs in various solid cancers including liver cancer started growing exponentially in a recent decade (Mishra et al. 2009; Ajani et al. 2015). Among a number of stemness markers highlighting hepatic stem/progenitor cells (HSPCs), CD133 and epithelial cell adhesion molecule (EpCAM) are the two most extensively investigated markers (Mishra et al. 2009; Grosse-Gehling et al. 2013; Dolle et al. 2015). This chapter starts by overviewing the concepts of stem cell, HSPC, and hepatic stemness markers. It is then followed by reviewing structures and biology of CD133 and EpCAM and finally concluded by clinical implications of CD133 and EpCAM in liver cancers.

Hepatic Stem/Progenitor Cells, Hepatic Cancer Stem Cells, and Hepatic Stemness Markers

In the concept of stem cell biology, stem cells, progenitor cells, and differentiated cells are three essential cellular components (Fig. 1; Table 1) (Clarke et al. 2006; Zhang et al. 2008). Stem cells are undifferentiated primitive cells with unlimited abilities of self-renewal, replication, and pluripotent differentiation. Self-renewal is different from replication/proliferation and characterized by production of one or two daughter cells with same abilities of replication and differentiation as the parental cells. Stem cells can undergo symmetric self-renewal cell division resulting in two identical daughter stem cells or asymmetric cell division producing one daughter stem cell and one more differentiated progenitor cell (Clarke et al. 2006). Progenitor cells, also known as transit-amplifying cells, are differentiated from stem cells, can undergo a limited number of replications, and finally differentiate into specialized cells. Specialized cells, also known as terminally differentiated cells, do

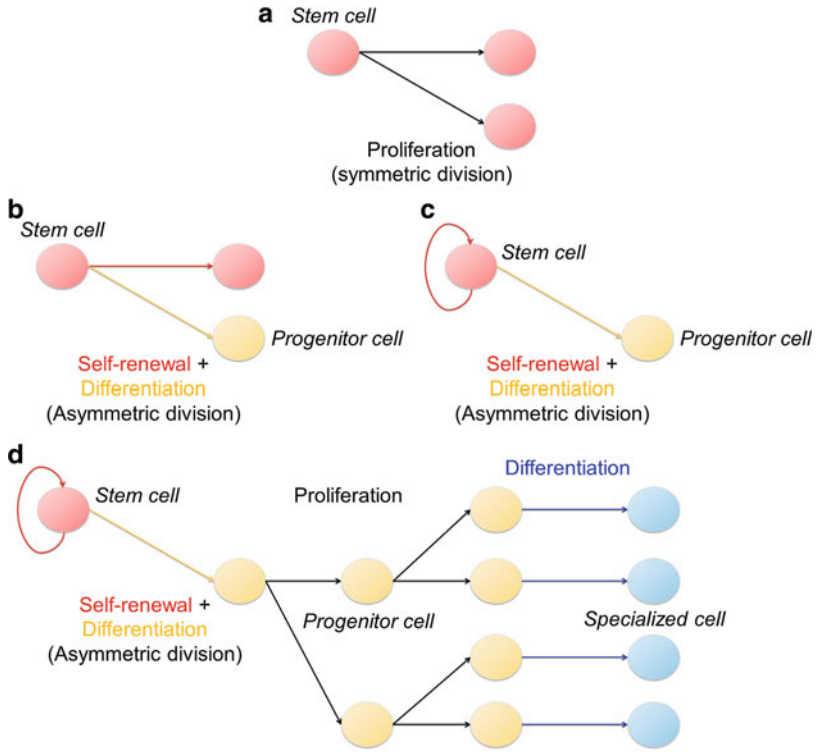

Fig. 1 Stem cells and their descendants. (**a**) Symmetric self-renewal cell division of a stem cell into two identical daughter stem cells. (**b**) Asymmetric self-renewal cell division of a stem cell into a daughter stem cell and a daughter progenitor cell. (**c**) Another notation of asymmetric self-renewal cell division of a stem cell. (**d**) Proliferation and differentiation of progenitor cells to specialized cells

Table 1 Comparison among stem cells, progenitor cells, and specialized cells

Properties	Stem cells	Progenitor cells	Specialized cells
Normal			
Self-renewal	Yes (unlimited)	Yes (limited)	No
Proliferation	Yes	Yes	No
Differentiation	Yes (pluripotent)	Yes (oligopotent)	No
Cancerous			
Immortality	Yes	No	No
Tumorigenicity	Yes	No	No
Resistance to chemo-/radiotherapy	Yes	No	No

not divide (or rarely divide) or differentiate anymore. Stem cells are immortal with infinite lifelong lifespan, whereas progenitor and specialized cells have finite lifetime in terms of days to months. Stem cell and progenitor cell are two distinct cells conceptually but a combined term "stem/progenitor cell" frequently appears in the literature because these two types of cells are not always distinguishable in reality. The place where stem cells are located is called stem cell niche. Stem cell niche is not only an anatomic site but also a functional microenvironment tightly controlling stem cell activity through interaction with other cellular and extracellular components in the niche (Scadden 2006). Without such an anatomic and functional stem cell niche, isolated stem cells have restricted stemness functions.

Liver is an organ well-known for its huge regenerating capacity, which was even described by the ancient Greeks. In the Greek myth, Prometheus stole the secret of fire from the gods of Olympus and was punished by being chained on the Mount Caucasus where his liver was eaten by eagles every day but regenerated completely overnight. In rodent, after resecting two-thirds of liver by partial hepatectomy, the residual liver restored to the initial size in a week (Taub 2004). Similarly, in human, after surgical removal of 40–50% of liver from a donor for transplantation, the remaining donor liver regenerated to over 80% of the original size in a month (Akamatsu et al. 2006). Hepatocytes and cholangiocytes (biliary epithelial cells) represent terminally differentiated specialized parenchymal cells in liver and make up 60% and 3–5% of total liver cells, respectively. Both of them account for more than 80% of the liver volume (Taub 2004). When mature hepatocytes or cholangiocytes are damaged by acute or chronic hepatic injuries (e.g., viral hepatitis, alcohol, drugs, and surgery), HSPCs are activated to restore the parenchymal loss. HSPC niche is situated in the primitive ductal plate in fetal liver and in the canals of Hering at the periportal interface between hepatocytic parenchyma and portal tract in the adult liver. HSPCs are postulated to be hepatoblasts in fetus and cells of canals of Hering in adult (Roskams 2006). Other components of HSPC niche include hepatic stellate cells, Kupffer cells, endothelial cells, and extracellular matrix (Williams et al. 2014). Ductular reaction, featured by proliferation of bile ductules (cholangioles) associated with inflammatory infiltrate in the periportal region, is regarded as an evidence of HSPC activation commonly found in various hepatic diseases.

CSC is the malignant counterpart of normal stem cell and defined as a small subpopulation of tumor cells resembling normal stem cells with unlimited abilities of self-renewal, replication, and pluripotent differentiation (Clarke et al. 2006). However, one should notice that CSCs may not be necessarily cancerous stem cells. It is still debatable on whether CSCs are originated from normal stem cells undergoing malignant transformation or a subpopulation of cancer cells acquiring stemness properties (Nguyen et al. 2012). CSCs are not only responsible for tumor initiation, heterogeneity, relapse, metastasis, and resistance to chemo-/radiotherapy but also are potential druggable targets for cancer treatment (Clarke et al. 2006; Nguyen et al. 2012; Ajani et al. 2015). Since isolation of hepatic CSCs from liver cancer cell lines was pioneered by two independent Japanese groups in 2006, (Chiba et al. 2006; Haraguchi et al. 2006) studies on CSCs in liver cancers have dramatically increased.

A number of markers have been proposed to identify HSPCs and hepatic CSCs and are designed as hepatic stem cell or stemness markers (Table 2) (Mishra et al. 2009; Ajani et al. 2015). HSPCs are characterized by the expression of certain hepatic stemness cells (e.g., CD133, cytokeratin 19 [CK19], EpCAM, and neural cell adhesion molecule [NCAM/CD56]) (Theise et al. 1999; Kim et al. 2011; Chan et al. 2014). Upon hepatocellular differentiation of HSPCs, hepatocytes lose the expression of CD133, CK19, EpCAM, and NCAM and gain the expression of HepPar-1, arginase-1, and polygonal carcinoembryonic antigen (canalicular pattern). On the other hand, toward biliary differentiation of HSPCs, cholangiocytes retain the expression of CK19 and EpCAM and lose the expression of CD133 and NCAM. Two important reminders for hepatic stemness markers should be noticed. First, these stemness markers are not entirely specific for HSPCs or hepatic CSCs but may be expressed in other normal and malignant cells in liver (e.g., CK19 and EpCAM are expressed in HSPCs, mature cholangiocytes, and cholangiocarcinoma). Second, none of these markers is the best to represent HSPCs. In two studies of CSCs in HCC, we and a Korean group showed that expressions of CD133, CK19, and

Table 2 Putative stemness markers in liver

Cell surface markers	Transcription factors
ABCG2	NANOG
ALDH	Oct4
CD13	SALL4
CD24	
CD44	
CD47	
CD56/NCAM	
CD90	
CD117/c-kit	
CD133	
CK19	
DLK1	
EpCAM	
OV6	

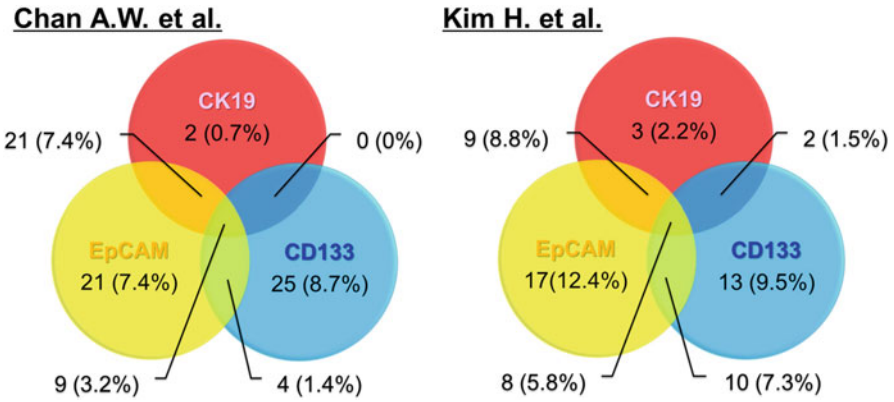

Fig. 2 Venn diagrams showing frequencies of CD133, CK19, and EpCAM expression in hepatocellular carcinoma (Kim et al. 2011; Chan et al. 2014)

EpCAM were positively correlated and partially overlapped with each other (Fig. 2) (Kim et al. 2011; Chan et al. 2014). HCC cells co-expressing more than one hepatic stemness markers exhibited more conspicuous stemness features (tumorigenicity and resistance to chemotherapy) than those expressing a single stemness marker only (Ma et al. 2008a; Zhu et al. 2010).

Structure and Biology of CD133

Human CD133 is encoded by PROM1 gene on chromosome 4p15.32, which contains 37 exons spanning about 141.3 kb. Its mRNA transcript is about 3.8–4.4 kb in size and has at least splice variants. CD133 is a pentaspan transmembrane single-chain glycoprotein, composed of 834–865 amino acids with weight of about 115–120 kDa (Cunningham et al. 2015). It consists of an N-terminal extracellular domain, five transmembrane domains, two large extracellular loops, two small intracellular loops, and a C-terminal intracytoplasmic tail. It has four potential N-glycosylation sites on each extracellular loop. The physiological function of CD133 remains unclear but its preferential localization in highly curved plasma membrane protrusions (e.g., primary cilia and microvilli) and close association with exosomes raise a possible role of CD133 in intercellular communication (Grosse-Gehling et al. 2013).

After CD133 was first identified as a hematopoietic stem cell marker in 1997, CD133 is regarded as a putative marker highlighting normal stem cells and CSCs of various tissues and organs including brain, colon, liver, pancreas, etc. (Grosse-Gehling et al. 2013; Ma 2013; Ajani et al. 2015). Several groups suggested that CD133-positive cells in liver are HSPCs participating in hepatic regeneration. Ma S. et al. used partial hepatectomy mouse model by removing over 70% of liver to illustrate the role of CD133 in liver regeneration secondary to acute injury. They showed that CD133 was markedly elevated in early regeneration (day 3 post-partial

hepatectomy) and significantly reduced in late stage (day 7 post-partial hepatectomy) (Ma et al. 2007). Fujii T et al. employed carbon tetrachloride mouse model to demonstrate that CD133 was involved in regeneration in chronic liver injury. They found persistent and dramatic elevation of CD133 after 4–6 weeks and 15–17 weeks of oral treatment of carbon tetrachloride and significant drop in 7–13 weeks after cessation of carbon tetrachloride (Fujii et al. 2010). Tsuchiya A. et al. revealed the presence of CD133-positive cells in ductular reaction in both acute and chronically damaged human livers (Tsuchiya et al. 2009). In addition to a HSPC marker, CD133 is proposed to be a marker of CSCs of liver cancers. CD133 was first recognized as a hepatic CSC marker in Huh-7 HCC cell line by Suetsugu A. et al. in 2006 (Suetsugu et al. 2006). Their in vitro and in vivo studies showed that CD133-positive Huh-7 cells had significantly higher ability of self-renewal and tumor initiation than CD133-negative Huh-7 cells. Moreover, CD133-positive Huh-7 cells were less differentiated than CD133-negative ones because they had lower expression of mature hepatocyte markers (glutamine synthetase and cytochrome P450 3A4) and higher expression of alpha fetoprotein (AFP). Subsequently, other groups confirmed high tumorigenicity and clonogenicity of CD133-positive HCC cells by in vitro and in vivo models (Ma et al. 2007; Yin et al. 2007). Ma S. et al. suggested chemoresistance of CD133-positive cells, which was evident by enrichment of a subpopulation of CD133-positive cells in HCC cell lines and xenograft mice after conventional chemotherapy (doxorubicin and fluorouracil) (Ma et al. 2008b). Co-expression of other putative hepatic CSC markers (e.g., aldehyde dehydrogenase, CD44, CK19, EpCAM, NCAM) could be found in CD133-positive HCC cells (Ma et al. 2008a; Zhu et al. 2010; Kim et al. 2011; Chan et al. 2014). Studies using immunohistochemistry reported that 0–40.0% (mean 22.0 $\pm$ 12.1%) of human HCC expressed CD133, and those CD133-positive HCCs were associated with higher histological grade and tumor stage (Chan et al. 2014).

Several aberrant signaling pathways are involved in CD133-positive hepatic cancers:

(a) Hedgehog signaling pathway: Proliferation of CD133-positive Huh-7 HCC cells is abolished by a specific hedgehog inhibitor, cyclopamine (Chen et al. 2011). The activity of hedgehog pathway was higher CD133-positive mouse HCC cells than CD133-negative ones, reflected by differential expression levels of sonic hedgehog and smoothened homologue (Jeng et al. 2013).
(b) Mechanistic target of rapamycin (mTOR) signaling pathway: mTOR, also known as mammalian target of rapamycin, regulated CD133 expression (Yang et al. 2011). Inhibition of mTOR by rapamycin enriched CD133-positive subpopulation and enhanced stemness features in HCC cell lines (Hep3B, Huh7, and PLC/PRF/5) in vitro and increased CD133-positive subset and facilitated re-propagation of Ras-dependent mouse liver tumors in vivo. In contrast, activation mTOR by overexpressing Rheb depleted CD133 subpopulation through differentiation of CD133-positive cells into CD133-negative cells.
(c) Interleukin-8 (IL-8)/chemokine C-X-C motif ligand 1 (CXCL1) signaling pathway: Tumorigenesis of CD133 was associated with deregulated neurotensin-induced

IL-8/CXCL1 and mitogen-activated protein kinase (MAPK)/Erk signaling pathways (Tang et al. 2012). CD133-positive HCC cells (Huh7 and PLC8024) had preferential expression of IL-8 mediated through positive feedback loop by MAPK pathway initiated by neurotensin. IL-8 promoted tumor self-renewal, proliferation, and angiogenesis of CD133-positive hepatic CSCs. Knockdown of CD133 repressed IL-8/CXCL1 cascade and obliterated stemness properties.

(d) Bone morphogenetic protein (BMP)/Smad signaling pathway: High-dose exogenous BMP4 activated canonical BMP/Smad pathway and induced Erk1/2 phosphorylation, resulting in promotion of differentiation and inhibition of stemness features (self-renewal, tumor initiation, and resistance to chemotherapy) of CD133-positive HCC cells (Huh7 and PLC/PRF/5). In contrast, low-dose or endogenous BMP4 was indispensable to maintain CD133-positive CSCs (Zhang et al. 2012a).

(e) MAPK/Erk signaling pathway: CD133 expression was regulated through crosstalk of MAPK/Erk pathway with IL8/CXCL1 and BMP/Smad pathways (Tang et al. 2012; Zhang et al. 2012b).

(f) Hypoxia-inducible factor 1-α/Notch signaling pathway: Annexin A3 was required for maintenance of CD133-positive hepatic CSCs through hypoxia-inducible factor 1-α/Notch pathway (Pan et al. 2015). The expression of annexin A3 was positively correlated with the proportion of CD133-positive cells and tumorigenicity in HCC cell lines (Hep3B, HepG2, and SMMC-7721). In HCC samples from patients, expression levels of annexin A3 and CD133 were also positively correlated and associated with tumor progression.

(g) Chemoresistance of CD133-positive HCC was mediated through activation of Akt and Bcl-2 survival pathways and insulin-like growth factor 1 receptor (Ma et al. 2008b; Bodzin et al. 2012), whereas radioresistance was associated with activation of MAPK/Erk pathway and inhibition of reactive oxygen species production (Piao et al. 2012). Moreover, the expression of phospho-c-Jun, which indicated the activity of c-Jun N-terminal kinase (JNK) in HCC specimens from patients receiving sorafenib, was associated with CD133 expression, enhanced tumor-initialing capacity of CD133-positive cells, and, more importantly, chemoresistance to sorafenib (Hagiwara et al. 2012).

(h) Last but not least, epigenetic modifications also participate in regulation of CD133 expression in liver cancers. Demethylation of the repetitive long interspersed nucleotide element-1 (Line-1) in HCC, signifying global hypomethylation, was associated with elevated CD133 expression (Zhang et al. 2011). Transforming growth factor-β (TGF-β) promoted CD133 expression through inhibition of DNA methyltransferase (DNMT) 1 and DNMT3β and subsequent demethylation of promoter-1 of CD133 (You et al. 2010). Both microRNA (miR)-130b and miR-155 upregulated CD133 expression and promoted epithelial-mesenchymal transition through silencing tumor protein 53-induced nuclear protein 1 (TP53INP1) (Ma et al. 2010; Liu et al. 2015). On the other hand, miR142-3p and miR-150 suppressed CD133 expression. The former is mediated by directly targeting CD133, whereas the latter is regulated through inhibition of transcription factor c-Myb (Zhang et al. 2012a; Chai et al. 2014).

Structure and Biology of EpCAM

Human EpCAM, also known as CD326 or trophoblast cell-surface antigen 1 (TROP1), is encoded on chromosome 2p21, which contains 15 exons spanning about 42 kb. Its mRNA transcript is about 609–1,724 base pairs in size and has four splice variants. EpCAM is a type I membrane glycoprotein composed of 199–342 amino acids with weight of about 30–40 kDa (Cunningham et al. 2015). It consists of an N-terminal extracellular domain (EpEx), a single transmembrane domain, and a C-terminal intracytoplasmic domain (EpICD). The extracellular domain contains epidermal growth factor-like and thyroglobulin repeat-like domains, and three potential N-glycosylation sites (Dolle et al. 2015). Although EpCAM is one of cell adhesion molecules (CAMs), it is structurally different from four major families of CAMs (cadherin, integrin, immunoglobulin family, and selectin) and its ability of intercellular adhesion is weak compared to E-cadherin. Expression levels of EpCAM and cadherin in epithelial cells are inversely correlated. EpCAM diminished the strength of cadherin-mediated intercellular interactions, abolished the cytoskeleton-bound fraction of the cadherin/α-catenin complex, and remodeled thickness and orientation of the actin filaments (Winter et al. 2003). The exact role of EpCAM contributing in tissue integrity is inconclusive. The function of EpCAM is complex and not restricted to cell-cell adhesion. EpCAM regulates proliferation, differentiation, migration, and cell survival through regulated intramembranous proteolysis. Sequential cleavage of EpCAM by tumor necrosis-factor alpha converting enzyme and gamma-secretase complex containing presenilin 2 releases EpEx and EpICD to participate in intercellular communication and intracellular interaction with Wnt/β-catenin signaling pathway, respectively (Dolle et al. 2015). EpCAM also interacts with Hippo/Yes-associated protein and Notch signaling pathways.

EpCAM is widely expressed in epithelial cells, normal stem cells, carcinoma cells, and CSCs of various tissues (Dolle et al. 2015). de Boer C.J. et al. first suggested that EpCAM is a stemness marker of HSPCs in 1999 (de Boer et al. 1999). They demonstrated that EpCAM highlighted hepatoblasts in 8-week embryonic liver and ductular reaction in hepatitis B-related cirrhosis, primary biliary cirrhosis, and focal nodular hyperplasia. Variation of EpCAM-positive cells in human liver of different chronological age was illustrated in detail by Zhang L. et al. (2008). In 18-week fetal livers, EpCAM was expressed in ductal plate cells with membranous and cytoplasmic staining and hepatoblasts with membranous staining throughout the developing hepatic lobule. In neonatal livers, ductal plate cells still expressed EpCAM in membranous and cytoplasm pattern but mature hepatocytes lost EpCAM staining. In pediatric and adult livers, EpCAM was confined to cells of canals of Hering only. The percentage of EpCAM-positive cells dropped from more than 80% in fetal liver to less than 0.01% in adult livers. In addition, the presence of EpCAM-positive HSPCs in ductular reaction in both acute (massive hepatic necrosis) and chronic (cirrhosis due to viral hepatitis, alcohol, and biliary disease) liver injuries was also demonstrated. Although de Boer C.J. et al. first documented the majority of IHCC (91%, 9/11) and a small subset (20%, 2/10) of

HCC expressed EpCAM, they only postulated the association between hepatic CSCs and IHCC (de Boer et al. 1999). Yamashita T et al. explicitly demonstrated that EpCAM-positive HCC had stemness features by gene expression and pathway analyses on tissue samples from HCC patients. They further illustrated that EpCAM-positive HCC Huh-7 cells were hepatic CSCs with tumorigenic and invasive potentials by HCC cell lines and xenografts (Yamashita et al. 2009). Later, other groups supported high tumorigenicity and clonogenicity of EpCAM-positive HCC cells by in vitro and in vivo models (Kimura et al. 2010; Terris et al. 2010). EpCAM-positive HCC cells often co-expressed other hepatic CSC markers (e.g., CD133, CK19, NCAM) (Yamashita et al. 2009; Kim et al. 2011; Chan et al. 2014). Studies using immunohistochemistry showed that 15.9–48.7% (mean 32.4 ± 12.8%) of human HCCs expressed EpCAM, and those EpCAM-positive HCCs were associated with younger age, higher histological grade, vascular invasion, and more advanced tumor stage (Chan et al. 2014).

Wnt/β-catenin signaling pathway is the major pathway regulating EpCAM expression in liver cancers. Activation of Wnt/β-catenin pathway by inhibiting glycogen synthase kinase-3β activity induced EpCAM expression, whereas blockage of Wnt/β-catenin cascade by accelerated degradation of β-catenin suppressed EpCAM expression. EpCAM is hence suggested to be a transcriptional target of Tcf/β-catenin (Yamashita et al. 2007). MicroRNAs targeting Wnt/β-catenin pathway also controlled EpCAM expression. miR-181 upregulated EpCAM by targeting regulators of hepatic differentiation (caudal type homeobox transcription factor 2 and GATA binding protein 6) and an inhibitor of Wnt/β-catenin pathway (nemo-like kinase) (Ji et al. 2009). On the other hand, miR-148a and miR-214 reduced EpCAM expression. The former targeted activin A receptor type 1 (a key regulator in BMP pathway) and inhibited Wnt/β-catenin pathway (Li et al. 2015), while the latter targeted β-catenin directly or indirectly through downregulation of enhancer of zeste homologue (EZH2) (Xia et al. 2012). EZH2 is the functional enzymatic component of polycomb repressive complex 2 responsible for methylation activity and could activate Wnt/β-catenin pathway. Lentivirus-mediated EZH2 knockdown and pharmacological ablation of EZH2 significantly diminished the amount and tumorigenic ability of EpCAM-positive HCC cells (Chiba et al. 2012).

Clinical Applications of CD133 and EpCAM for Diagnosis and Prognosis in Liver Cancers

Combined Hepatocellular-Cholangiocarcinoma with Stem Cell Features

HCC and IHCC represent two extremes in the spectrum of malignant epithelial liver neoplasms in adult. Combined hepatocellular-cholangiocarcinoma (HCC-CC) is an intermediate tumor with unequivocal, intimately mixed components of both HCC and cholangiocarcinoma (Theise et al. 2010b). It is a rare tumor accounting for <1%

of all liver cancers and has age/sex-specific incidence and geographic variations similar to those of HCC. Its prognosis is in between HCC and IHCC: better than HCC but worse than IHCC (Koh et al. 2005). With emergence of stemness marker, a new variant of HCC-CC, HCC-CC with stem cell features, was introduced in the latest fourth edition of the WHO classification, and the original HCC-CC was renamed as HCC-CC of classical type (Theise et al. 2010a).

HCC-CC with stem cell features are those tumors that have specific morphological features together with expression of stemness markers and can be classified into three major subtypes (Fig. 3a–c). The first subtype is typical subtype, which was first described by Theise N.D. et al. in 2003 (Theise et al. 2003). It is characterized by nests of mature-appearing hepatocytes surrounded by small oval primitive cells with high nucleocytoplasmic ratio and expression of stemness markers. The second subtype is intermediate subtype, which was initially reported by Kim H. et al. in 2004 (Kim et al. 2004). It is composed of trabeculae and solid nests of small oval primitive cells. The third subtype is cholangiocellular subtype, which was originally described by Steiner P.E. and Higginson J. in 1959 (Steiner and Higginson 1959). It was previously classified as a variant of IHCC in the 3rd edition of the WHO classification. It is featured by anastomosing tubules and cord-like structure of small oval primitive cells. Such a growth pattern recapitulates ductular reaction in normal liver. Those small oval primitive cells in all three subtypes exhibit high nucleocytoplasmic ratio and express various stemness markers. Different subtypes of HCC-CC with stem cell features are suggested to have different pathological significances. The cholangiocellular subtype was associated with smaller tumor size, better histological differentiation of coexisting HCC, and more severe inflammation, whereas the intermediate subtype was associated with larger size, poorer histological differentiation of coexisting HCC, and less intratumoral fibrosis (Sasaki et al. 2015).

There are two important clinical implications of HCC-CC with stem cell features. The first one is associated with therapeutic resistance of hepatic CSCs. The use of neoadjuvant therapy to downstage tumor before curative treatment is not uncommon in patients with HCC. Zen C. et al. showed that 25% of HCC underwent liver transplantation after neoadjuvant transarterial chemoembolization (TACE) exhibited transformation to HCC-CC with stem cell features: cholangiolar differentiation with stemness marker expression (Zen et al. 2011). This finding may indicate that neoadjuvant therapy may select or activate hepatic CSCs in HCC due to selection pressure of a chemoresistant subpopulation. The second one is the prognosis of this new variant. Compared to HCC-CC of classical type, the prognosis of HCC-CC with stem cell features is controversial. Akiba J. et al. showed that HCC-CC with stem cell features ($n = 44$) had similar overall and progression-free survival to HCC-CC of classical type ($n = 10$) (Akiba et al. 2013). Ikeda H. et al. found that HCC-CC with stem cell features ($n = 24$) had poorer overall survival than HCC-CC of classical type ($n = 12$) (Ikeda et al. 2013). Although larger cohorts with longer follow-up are required to further characterize the clinical outcome of this new variant, we could still conclude that HCC-CC with stem cell features has inferior outcome than classical HCC.

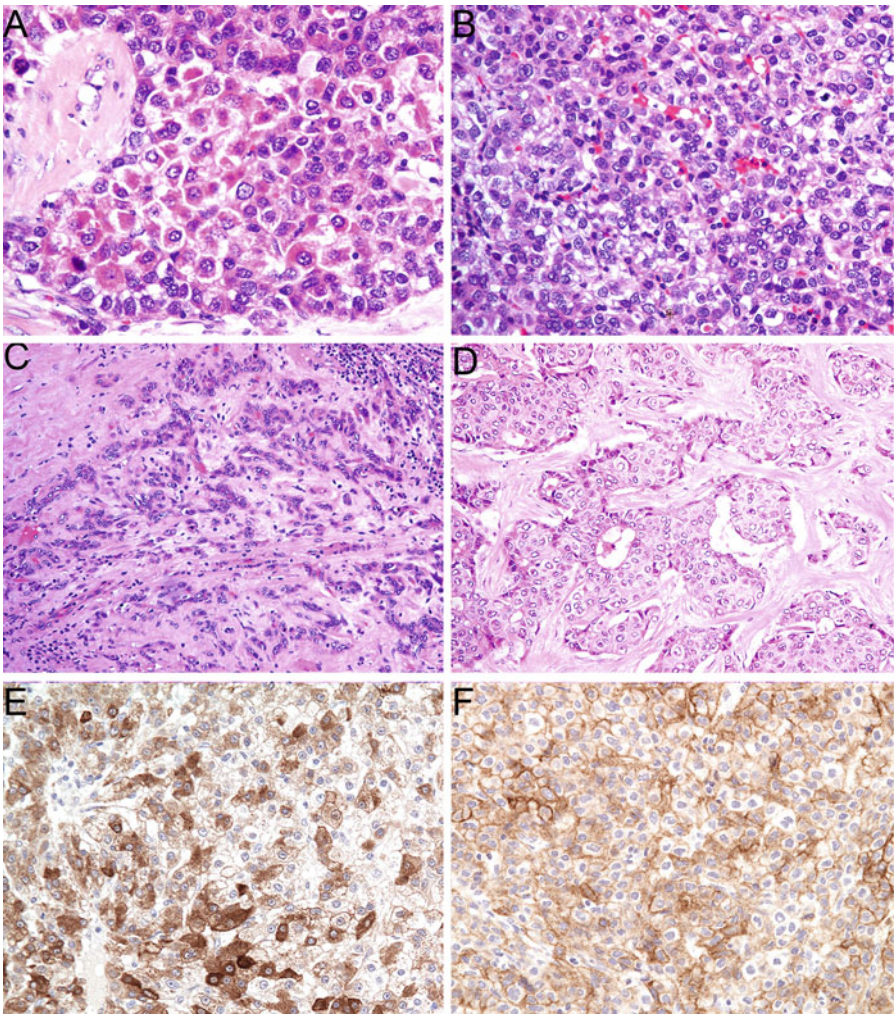

Fig. 3 Histology of (**a**) Combined hepatocellular-cholangiocarcinoma (HCC-CC) with stem cell feature, typical subtype (H&E, original magnification 400×); (**b**) HCC-CC with stem cell feature, intermediate subtype (H&E, original magnification 400×); (**c**) HCC-CC with stem cell feature, cholangiolar subtype (H&E, original magnification 200×); (**d**) Scirrhous hepatocellular carcinoma (H&E, original magnification 200×); (**e**) Hepatocellular carcinoma expressing stem cell features (CD133, original magnification 400×); (**f**) Hepatocellular carcinoma expressing stem cell features (EpCAM, original magnification 400×)

Scirrhous Hepatocellular Carcinoma

Scirrhous HCC is another well-described variant of HCC that was also found to have association with hepatic CSCs. It is an uncommon histological variant accounting for 5% of all HCCs (Theise et al. 2010b). It is characterized by trabeculae, nests, and

sheets of malignant hepatocytes embedded in marked fibrotic stroma (30–50% of tumor area) (Fig. 3d). It is similar to classical HCC clinically and cytologically. It is different from another well-known HCC variant, fibrolamellar HCC, by absence of large polygonal eosinophilic cells with abundant cytoplasm and pale bodies and characteristic lamellar fibrous bands. Our group compared 37 scirrhous HCC and 309 classical HCC from a cohort of HCC underwent surgical resection. We demonstrated that scirrhous HCC had more frequent major vessel invasion (21.6% vs. 9.4%; $P = 0.042$), higher median serum AFP (149 μg/l vs. 60 μg/l; $P = 0.020$), higher Barcelona clinic liver cancer (BCLC) stage (stage B/C/D 45.9% vs. 27.5%; $P = 0.020$), worse median disease-free survival (23.3 months vs. 73.0 months, $P = 0.032$), and higher expression of CD133/EpCAM (30.4% vs. 12.7%; $P < 0.001$) (Unpublished data). Our findings concurred with those reported by Seok J.Y. et al.: Scirrhous HCC was associated with portal vein invasion, microvascular invasion, poorer disease-free survival, and more frequent stemness marker expression (Seok et al. 2012). They also demonstrated activation of epithelial-mesenchymal transition and TGF-β signal pathway in scirrhous HCC, which could be linked up with hepatic CSCs.

Hepatocellular Carcinoma Expressing Stemness Markers

HCC expressing stemness markers was described in the latest fourth edition of the WHO classification. This variant has morphological features of classical HCC together with expression of stemness markers in more than 5% of tumor cells (Fig. 3e, f) (Theise et al. 2010a). Actually, HCCs aberrantly exhibiting stemness markers have been described for many years. Van Ekyen P. et al. first described 50% (17/34) of HCC with abnormal expression of "biliary-type" cytokeratin 19 (Van Eyken et al. 1988). de Boer C.J. et al. also reported a small subset (2/10, 20%) of HCC expressed EpCAM (de Boer et al. 1999). Our group investigated a cohort of 284 patients with primary HCC receiving curative surgical resection and revealed that 31.0% of HCC expressed one or more stemness markers (CD133, CK19, EpCAM, and NCAM) (Chan et al. 2014). This histological HCC variant is associated with younger age, cirrhotic background, smaller tumor size, and higher serum AFP. It had worse prognosis than classical HCC: median overall survival (122.5 months vs. not reached; $P = 0.028$) and disease-free survival (22.0 months vs. 98.9 months; $P < 0.001$). These findings were consistent with those described by other groups (Roskams 2006; Kim et al. 2011).

For the prognostic value of CD133, we found that CD133 expression was an independent prognostic factor associated with poorer overall (hazard ratio 2.30; $P < 0.001$) and disease-free survival (hazard ratio 1.86; $P = 0.006$) in addition to American Joint Committee on Cancer (AJCC) tumor stage (Chan et al. 2014). We are the first group to establish that CD133 was an independent factor predicting poorer overall (hazard ratio 3.91; $P = 0.001$) and disease-free (hazard ratio 2.82; $P = 0.001$) survival among HCC patients with AJCC stage I disease. The prognostic

significance of CD133 has been also validated by other studies (Ma et al. 2013). The prognostic implication of EpCAM expression is controversial (Ma et al. 2013). Bae J.S. et al. first suggested that EpCAM-positive HCC had significantly worse outcome among HCC patients with AJCC stage I disease (Bae et al. 2012). However, their definition of AJCC stage I was questionable because 8.3% of patients had intrahepatic metastases and should be properly recategorized to higher tumor stage. Our study revealed that EpCAM expression was an independent prognostic factor to predict worse disease-free survival (hazard ratio 2.05; $P = 0.001$) in addition to American Joint Committee on Cancer (AJCC) tumor stage and CD133 (Chan et al. 2014). We also showed that CD133 was an independent factor predicting poorer overall (hazard ratio 3.85; $P = 0.001$) and disease-free (hazard ratio 3.22; $P = 0.001$) survival among HCC patients with AJCC stage III/IV disease. Induction of expression of CD133 and EpCAM in HCC after neoadjuvant TACE was observed, and high expression levels of CD133 and EpCAM were associated with tumor recurrence after liver transplantation following TACE (Zen et al. 2011; Zeng et al. 2012). The prognostic importance of EpCAM is not only confined to the tissue level but also valid in peripheral blood. Sun Y.F. et al. found that the presence of EpCAM-positive circulating tumor cells in peripheral blood of HCC patients was associated with higher tumor relapse (70.6% vs. 20.8%; $P < 0.001$) and shorter recurrence-free survival (4.9 months vs. not reached; $P < 0.001$). The presence of EpCAM-positive circulating tumor cells was also correlated with higher tumor multiplicity, vascular invasion, poorer histological differentiation, and higher serum AFP (Sun et al. 2013).

In summary, those HCC variants closely related to stemness markers (HCC-CC with stem cell features, scirrhous HCC, and HCC expressing stemness markers) contribute to a new diagnostic spectrum of primary liver cancers (Fig. 4). All of them have worse clinical outcome than classical HCC. To establish the diagnosis of HCC-CC with stem cell features and HCC expressing stemness markers, the application of one or more stemness markers are essential.

Intrahepatic Cholangiocarcinoma and Hepatoblastoma

In contrast to HCC, there are only few studies of CD133 and EpCAM in prognostication of IHCC and hepatoblastoma, and their results are conflicting. Shimada M. et al. reported that 48.2% (14/29) of IHCC expressed CD133 and CD133 expression was an independent prognostic factor for overall survival (hazard ratio 3.19; $P = 0.038$) (Shimada et al. 2010). Thanan R. et al. showed that 56% (19/34) of IHCC harbored CD133 and those tumors with expression of CD133 or another stemness marker (Oct3/4) was associated with smaller tumor size, more frequent tubular configuration, more advanced stage, and poor prognosis (Thanan et al. 2013). However, Fan L. et al. found that 74% (40/54) of cholangiocarcinoma showing CD133 positivity. Among 23 patients with follow-up data, CD133-positive tumors had better prognosis than CD133-negative ones (median survival of

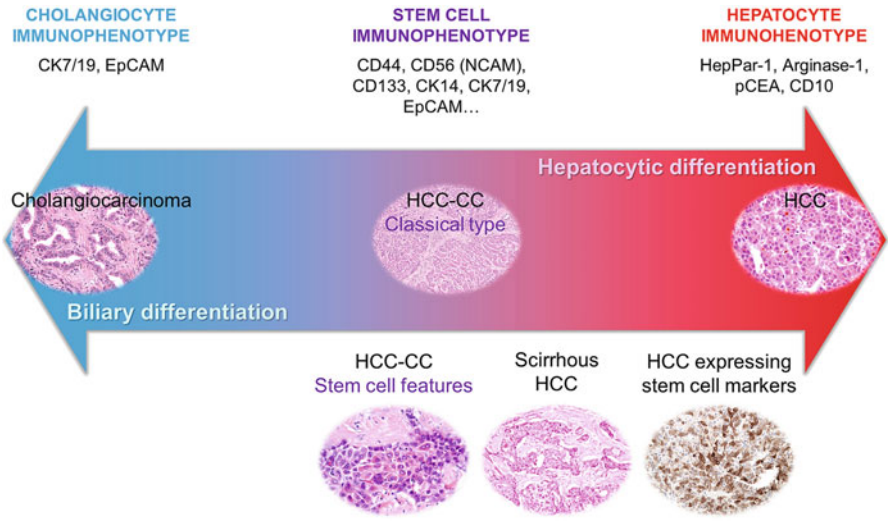

Fig. 4 The diagnostic spectrum of primary liver cancers in adult according to their histological appearance and immunophenotypes

14 months vs. 4 months; $P = 0.001$). One should be noticed that cholangiocarcinoma in this study was composed of both intrahepatic (25/54) and hilar (29/54) tumors and these two distinct types of cholangiocarcinoma were analyzed as a single group (Fan et al. 2011). As normal cholangiocytes and most of malignant cholangiocytes in IHCC express EpCAM, (de Boer et al. 1999) the prognostic application of EpCAM in IHCC is seldom investigated. Sulpice L. et al. recently described that intra-tumorous stromal expression (rather than epithelial expression) of EpCAM in IHCC was significantly overexpressed than in non-tumorous fibrous tissue. Intra-tumorous stromal EpCAM expression was an independent prognostic factor associated with inferior overall (hazard ratio 2.62; $P = 0.005$) and disease-free (hazard ratio 2.23; $P = 0.012$) survival (Sulpice et al. 2014).

Bahnassy A.A. et al. revealed that CD133 was expressed in 48.8% (21/43) of hepatoblastoma and associated with fetal histological subtype, more advanced stage, higher serum AFP, and poorer treatment response (Bahnassy et al. 2015). CD133-positive hepatoblastoma had worse 4-year overall (0% vs. 95.4%; $P < 0.001$) and disease-free (53.3% vs. 88.8%; $P = 0.030$) survival than CD133-negative hepatoblastoma. However, CD133 was not an independent prognostic marker. Indeed, other two stemness markers, CD90 and CD44, were independent prognostic factors for overall and disease-free survival, respectively. Yun W.J. documented that 83.6% (51/61) of hepatoblastoma showed EpCAM immunopositivity, which was associated with higher serum AFP. However, there was not any correlation of EpCAM immunoreactivity with other clinicopathological characteristics and clinical outcome (Yun et al. 2013).

Clinical Applications of CD133 and EpCAM for Therapy in Liver Cancers

Sorafenib has been considered as the standard systemic agent for patients with advanced HCC since 2008. Despite different clinical trials on various new targeted agents afterwards, none of these targeted agents are better than sorafenib in the first-line setting or superior than placebo in the second-line setting (Chan et al. 2015). Moreover, there is no definite biomarker to predict therapeutic response to sorafenib. As previously mentioned, Hagiwara et al. demonstrated that high expression of phospho-c-Jun was associated with CD133 expression and resistance to sorafenib therapy. Tumor with high CD133 expression more frequently developed disease progression than those with low CD133 expression (27% vs. 64%, $P = 0.027$) (Hagiwara et al. 2012). Further investigations are required to determine whether CD133 can be used as a negative surrogate marker predicting clinical response to sorafenib.

Although effective direct therapy targeting hepatic CSCs is still lacking in clinical practice, a number of agents are being explored in preclinical settings. Smith L.M. et al. targeted CD133-positive liver cancer cell lines (Hep3B) by using an anti-CD133 antibody conjugated to a potent chemotherapeutic agent (monomethyl auristatin). They found significant suppression of Hep3B cell proliferation in vitro with IC50 value of 2.2 ng/ml and dramatic inhibition of Hep3B tumor growth in immunodeficient mice in vivo (Smith et al. 2008). Several agents targeting EpCAM are in clinical development. Catumaxomab is a monoclonal bispecific antibody against EpCAM and CD3 and has been approved in the European Union to treat EpCAM-positive carcinoma-associated malignant ascites. Two other EpCAM targeted antibodies, VB4-845 and adecatumumab, have been also tested in phase II/III trials for different non-hepatic carcinomas (Simon et al. 2013). Ogawa K. et al. showed that VB4-845 in combination with 5-fluorouracil inhibited proliferation of EpCAM-positive liver cancer cells (HepG2, Hep3B, Huh-1, HuH-7) in vitro and dramatically reduced the tumor volume in subcutaneous and orthotopic liver xenograft models in vivo (Ogawa et al. 2014). The preclinical findings of anti-CD133 and anti-EpCAM are promising but proper clinical trials on human subjects are crucial.

Conclusion

Through this chapter, the concepts of stem cell, HSPC, and hepatic stemness markers have been briefly introduced. Structures and biology of CD133 and EpCAM with emphasis on HSPC and liver cancers have been reviewed. Understanding expression and regulation of hepatic stemness markers (CD133 and EpCAM) is not only an academic interest in basic research but also carries clinical implications among patients with liver cancers. Utilization of CD133 and/or EpCAM is essential in

diagnosing certain distinct variants of HCC with clinical importance. CD133 and EpCAM are also important prognostic biomarkers predicting clinical outcomes of patients with liver cancers. Targeted therapies against CD133 and EpCAM are promising new therapeutic research areas to combat the key player, CSC, responsible for tumor recurrence and therapeutic resistance in deadly liver cancers.

Summary Points

- Stem cells are undifferentiated primitive cells with unlimited abilities of self-renewal, replication, and pluripotent differentiation.
- Cancer stem cells are a small subpopulation of tumor cells with features resembling normal stem cells including unlimited abilities of self-renewal, replication, and pluripotent differentiation and are responsible for tumor initialization, heterogeneity, relapse, metastasis, and resistance to chemo/radiotherapy but also are potential druggable targets for cancer treatment.
- CD133 and EpCAM are putative markers of stem/progenitor cells and cancer stem cells in liver.
- CD133 and EpCAM are crucial in diagnosing certain distinct variants of hepatocellular carcinoma with clinical importance: Combined hepatocellular-cholangiocarcinoma with stem cell features and hepatocellular carcinoma expressing stemness markers.
- CD133 and EpCAM are independent prognostic factors of overall and disease-free survival for hepatocellular carcinoma.
- Induction of expression of CD133 and EpCAM in hepatocellular carcinoma after neoadjuvant therapy is associated with tumor recurrence.
- The prognostic roles of CD133 and EpCAM in intrahepatic cholangiocarcinoma and hepatoblastoma have not been clearly defined yet.
- Targeted therapies against CD133 and EpCAM against liver cancers are still in preclinical stage, but they are promising new therapeutic research areas to combat the key player, cancer stem cell, responsible for tumor recurrence, and therapeutic resistance liver cancers.

References

Ajani JA, Song S, Hochster HS, et al. Cancer stem cells: the promise and the potential. Semin Oncol. 2015;42 Suppl 1:S3–17.

Akamatsu N, Sugawara Y, Tamura S, et al. Regeneration and function of hemiliver graft: right versus left. Surgery. 2006;139:765–72.

Akiba J, Nakashima O, Hattori S, et al. Clinicopathologic analysis of combined hepatocellular-cholangiocarcinoma according to the latest WHO classification. Am J Surg Pathol. 2013;37:496–505.

Bae JS, Noh SJ, Jang KY, et al. Expression and role of epithelial cell adhesion molecule in dysplastic nodule and hepatocellular carcinoma. Int J Oncol. 2012;41:2150–8.

Bahnassy AA, Fawzy M, El-Wakil M, et al. Aberrant expression of cancer stem cell markers (CD44, CD90, and CD133) contributes to disease progression and reduced survival in hepatoblastoma patients: 4-year survival data. Transl Res. 2015;165:396–406.
Bodzin AS, Wei Z, Hurtt R, et al. Gefitinib resistance in HCC mahlavu cells: upregulation of CD133 expression, activation of IGF-1R signaling pathway, and enhancement of IGF-1R nuclear translocation. J Cell Physiol. 2012;227:2947–52.
Chai S, Tong M, Ng KY, et al. Regulatory role of miR-142-3p on the functional hepatic cancer stem cell marker CD133. Oncotarget. 2014;5:5725–35.
Chan AW, Tong JH, Chan SL, et al. Expression of stemness markers (CD133 and EpCAM) in prognostication of hepatocellular carcinoma. Histopathology. 2014;64:935–50.
Chan SL, Chan AW, Yeo W. Novel therapeutic targets and predictive markers for hepatocellular carcinoma. Expert Opin Ther Targets. 2015;19:973–83.
Chen X, Lingala S, Khoobyari S, et al. Epithelial mesenchymal transition and hedgehog signaling activation are associated with chemoresistance and invasion of hepatoma subpopulations. J Hepatol. 2011;55:838–45.
Chiba T, Kita K, Zheng YW, et al. Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. Hepatology. 2006;44:240–51.
Chiba T, Suzuki E, Negishi M, et al. 3-Deazaneplanocin A is a promising therapeutic agent for the eradication of tumor-initiating hepatocellular carcinoma cells. Int J Cancer. 2012;130: 2557–67.
Clarke MF, Dick JE, Dirks PB, et al. Cancer stem cells – perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res. 2006;66:9339–44.
Cunningham F, Amode MR, Barrell D, et al. Ensembl 2015. Nucleic Acids Res. 2015;43:D662–9.
de Boer CJ, van Krieken JH, Janssen-van Rhijn CM, et al. Expression of Ep-CAM in normal, regenerating, metaplastic, and neoplastic liver. J Pathol. 1999;188:201–6.
Dolle L, Theise ND, Schmelzer E, et al. EpCAM and the biology of hepatic stem/progenitor cells. Am J Physiol Gastrointest Liver Physiol. 2015;308:G233–50.
Fan L, He F, Liu H, et al. CD133: a potential indicator for differentiation and prognosis of human cholangiocarcinoma. BMC Cancer. 2011;11:320.
Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11 [Internet]. 2012. Available at: http://globocan.iarc.fr. Accessed 20 Jan 2014.
Fujii T, Fuchs BC, Yamada S, et al. Mouse model of carbon tetrachloride induced liver fibrosis: histopathological changes and expression of CD133 and epidermal growth factor. BMC Gastroenterol. 2010;10:79.
Grosse-Gehling P, Fargeas CA, Dittfeld C, et al. CD133 as a biomarker for putative cancer stem cells in solid tumours: limitations, problems and challenges. J Pathol. 2013;229:355–78.
Hagiwara S, Kudo M, Nagai T, et al. Activation of JNK and high expression level of CD133 predict a poor response to sorafenib in hepatocellular carcinoma. Br J Cancer. 2012;106:1997–2003.
Haraguchi N, Utsunomiya T, Inoue H, et al. Characterization of a side population of cancer cells from human gastrointestinal system. Stem Cells. 2006;24:506–13.
Ikeda H, Harada K, Sato Y, et al. Clinicopathologic significance of combined hepatocellular-cholangiocarcinoma with stem cell subtype components with reference to the expression of putative stem cell markers. Am J Clin Pathol. 2013;140:329–40.
Jeng KS, Sheen IS, Jeng WJ, et al. Activation of the sonic hedgehog signaling pathway occurs in the CD133 positive cells of mouse liver cancer Hepa 1–6 cells. Oncol Targets Ther. 2013;6:1047–55.
Ji J, Yamashita T, Budhu A, et al. Identification of microRNA-181 by genome-wide screening as a critical player in EpCAM-positive hepatic cancer stem cells. Hepatology. 2009;50:472–480.
Kim H, Park C, Han KH, et al. Primary liver carcinoma of intermediate (hepatocyte-cholangiocyte) phenotype. J Hepatol. 2004;40:298–304.
Kim H, Choi GH, Na DC, et al. Human hepatocellular carcinomas with “Stemness”-related marker expression: keratin 19 expression and a poor prognosis. Hepatology. 2011;54:1707–17.

Kimura O, Takahashi T, Ishii N, et al. Characterization of the epithelial cell adhesion molecule (EpCAM)+ cell population in hepatocellular carcinoma cell lines. Cancer Sci. 2010;101: 2145–55.
Koh KC, Lee H, Choi MS, et al. Clinicopathologic features and prognosis of combined hepatocellular cholangiocarcinoma. Am J Surg. 2005;189:120–5.
Li L, Liu Y, Guo Y, et al. Regulatory MiR-148a-ACVR1/BMP circuit defines a cancer stem cell-like aggressive subtype of hepatocellular carcinoma. Hepatology. 2015;61:574–584.
Liu F, Kong X, Lv L, et al. TGF-beta1 acts through miR-155 to down-regulate TP53INP1 in promoting epithelial-mesenchymal transition and cancer stem cell phenotypes. Cancer Lett. 2015;359:288–98.
Ma S. Biology and clinical implications of CD133(+) liver cancer stem cells. Exp Cell Res. 2013;319:126–32.
Ma S, Chan KW, Hu L, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology. 2007;132:2542–56.
Ma S, Chan KW, Lee TK, et al. Aldehyde dehydrogenase discriminates the CD133 liver cancer stem cell populations. Mol Cancer Res. 2008a;6:1146–53.
Ma S, Lee TK, Zheng BJ, et al. CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. Oncogene. 2008b;27:1749–58.
Ma S, Tang KH, Chan YP, et al. miR-130b promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. Cell Stem Cell. 2010;7:694–707.
Ma YC, Yang JY, Yan LN. Relevant markers of cancer stem cells indicate a poor prognosis in hepatocellular carcinoma patients: a meta-analysis. Eur J Gastroenterol Hepatol. 2013;25: 1007–16.
Mishra L, Banker T, Murray J, et al. Liver stem cells and hepatocellular carcinoma. Hepatology. 2009;49:318–29.
Nakashima O, Curado MP, Franceshi S, et al. Intrahepatic cholangiocarcinoma. In: Bosman FT, Carneiro F, Hruban RH, et al., editors. WHO classification of tumours of the digestive system. Lyon: International Agency for Research on Cancer; 2010. p. 217–24.
Nguyen LV, Vanner R, Dirks P, et al. Cancer stem cells: an evolving concept. Nat Rev Cancer. 2012;12:133–43.
Ogawa K, Tanaka S, Matsumura S, et al. EpCAM-targeted therapy for human hepatocellular carcinoma. Ann Surg Oncol. 2014;21:1314–22.
Pan QZ, Pan K, Wang QJ, et al. Annexin A3 as a potential target for immunotherapy of liver cancer stem-like cells. Stem Cells. 2015;33:354–66.
Piao LS, Hur W, Kim TK, et al. CD133+ liver cancer stem cells modulate radioresistance in human hepatocellular carcinoma. Cancer Lett. 2012;315:129–37.
Roskams T. Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. Oncogene. 2006;25:3818–22.
Sasaki M, Sato H, Kakuda Y, et al. Clinicopathological significance of 'subtypes with stem-cell feature' in combined hepatocellular-cholangiocarcinoma. Liver Int. 2015;35:1024–35.
Scadden DT. The stem-cell niche as an entity of action. Nature. 2006;441:1075–9.
Seok JY, Na DC, Woo HG, et al. A fibrous stromal component in hepatocellular carcinoma reveals a cholangiocarcinoma-like gene expression trait and epithelial-mesenchymal transition. Hepatology. 2012;55:1776–86.
Shimada M, Sugimoto K, Iwahashi S, et al. CD133 expression is a potential prognostic indicator in intrahepatic cholangiocarcinoma. J Gastroenterol. 2010;45:896–902.
Simon M, Stefan N, Pluckthun A, et al. Epithelial cell adhesion molecule-targeted drug delivery for cancer therapy. Expert Opin Drug Deliv. 2013;10:451–68.
Smith LM, Nesterova A, Ryan MC, et al. CD133/prominin-1 is a potential therapeutic target for antibody-drug conjugates in hepatocellular and gastric cancers. Br J Cancer. 2008; 99:100–9.
Steiner PE, Higginson J. Cholangiolocellular carcinoma of the liver. Cancer. 1959;12:753–9.

Suetsugu A, Nagaki M, Aoki H, et al. Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. Biochem Biophys Res Commun. 2006;351:820–4.
Sulpice L, Rayar M, Turlin B, et al. Epithelial cell adhesion molecule is a prognosis marker for intrahepatic cholangiocarcinoma. J Surg Res. 2014;192:117–23.
Sun YF, Xu Y, Yang XR, et al. Circulating stem cell-like epithelial cell adhesion molecule-positive tumor cells indicate poor prognosis of hepatocellular carcinoma after curative resection. Hepatology. 2013;57:1458–68.
Tang KH, Ma S, Lee TK, et al. CD133(+) liver tumor-initiating cells promote tumor angiogenesis, growth, and self-renewal through neurotensin/interleukin-8/CXCL1 signaling. Hepatology. 2012;55:807–20.
Taub R. Liver regeneration: from myth to mechanism. Nat Rev Mol Cell Biol. 2004;5:836–47.
Terris B, Cavard C, Perret C. EpCAM, a new marker for cancer stem cells in hepatocellular carcinoma. J Hepatol. 2010;52:280–1.
Thanan R, Pairojkul C, Pinlaor S, et al. Inflammation-related DNA damage and expression of CD133 and Oct3/4 in cholangiocarcinoma patients with poor prognosis. Free Radic Biol Med. 2013;65:1464–72.
Theise ND, Saxena R, Portmann BC, et al. The canals of Hering and hepatic stem cells in humans. Hepatology. 1999;30:1425–33.
Theise ND, Yao JL, Harada K, et al. Hepatic 'stem cell' malignancies in adults: four cases. Histopathology. 2003;43:263–71.
Theise ND, Curado MP, Franceshi S, et al. Hepatocellular carcinoma. In: Bosman FT, Carneiro F, Hruban RH, et al., editors. WHO classification of tumours of the digestive system. Lyon: International Agency for Research on Cancer; 2010a. p. 205–16.
Theise ND, Nakashima O, Park YN, et al. Combined hepatocellular-cholangiocarcinoma. In: Bosman FT, Carneiro F, Hruban RH, et al., editors. WHO classification of tumours of the digestive system. Lyon: International Agency for Research on Cancer; 2010b. p. 225–7.
Tsuchiya A, Kamimura H, Takamura M, et al. Clinicopathological analysis of CD133 and NCAM human hepatic stem/progenitor cells in damaged livers and hepatocellular carcinomas. Hepatol Res. 2009;39:1080–90.
Van Eyken P, Sciot R, Paterson A, et al. Cytokeratin expression in hepatocellular carcinoma: an immunohistochemical study. Hum Pathol. 1988;19:562–8.
Williams MJ, Clouston AD, Forbes SJ. Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion. Gastroenterology. 2014;146:349–56.
Winter MJ, Nagelkerken B, Mertens AE, et al. Expression of Ep-CAM shifts the state of cadherin-mediated adhesions from strong to weak. Exp Cell Res. 2003;285:50–8.
Xia H, Ooi LL, Hui KM. MiR-214 targets beta-catenin pathway to suppress invasion, stem-like traits and recurrence of human hepatocellular carcinoma. PLoS One. 2012;7:e44206.
Yamashita T, Budhu A, Forgues M, et al. Activation of hepatic stem cell marker EpCAM by Wnt-beta-catenin signaling in hepatocellular carcinoma. Cancer Res. 2007;67:10831–9.
Yamashita T, Ji J, Budhu A, et al. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. Gastroenterology. 2009;136:1012–24.
Yang Z, Zhang L, Ma A, et al. Transient mTOR inhibition facilitates continuous growth of liver tumors by modulating the maintenance of CD133+ cell populations. PLoS One. 2011;6:e28405.
Yin S, Li J, Hu C, et al. CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. Int J Cancer. 2007;120:1444–50.
You H, Ding W, Rountree CB. Epigenetic regulation of cancer stem cell marker CD133 by transforming growth factor-beta. Hepatology. 2010;51:1635–44.
Yun WJ, Shin E, Lee K, et al. Clinicopathologic implication of hepatic progenitor cell marker expression in hepatoblastoma. Pathol Res Pract. 2013;209:568–73.
Zen C, Zen Y, Mitry RR, et al. Mixed phenotype hepatocellular carcinoma after transarterial chemoembolization and liver transplantation. Liver Transpl. 2011;17:943–54.
Zeng Z, Ren J, O'Neil M, et al. Impact of stem cell marker expression on recurrence of TACE-treated hepatocellular carcinoma post liver transplantation. BMC Cancer. 2012;12:584.

Zhang L, Theise N, Chua M, et al. The stem cell niche of human livers: symmetry between development and regeneration. Hepatology. 2008;48:1598–607.
Zhang C, Xu Y, Zhao J, et al. Elevated expression of the stem cell marker CD133 associated with Line-1 demethylation in hepatocellular carcinoma. Ann Surg Oncol. 2011;18:2373–80.
Zhang J, Luo N, Luo Y, et al. microRNA-150 inhibits human CD133-positive liver cancer stem cells through negative regulation of the transcription factor c-Myb. Int J Oncol. 2012a;40:747–56.
Zhang L, Sun H, Zhao F, et al. BMP4 administration induces differentiation of CD133+ hepatic cancer stem cells, blocking their contributions to hepatocellular carcinoma. Cancer Res. 2012b;72:4276–85.
Zhu Z, Hao X, Yan M, et al. Cancer stem/progenitor cells are highly enriched in CD133+CD44+ population in hepatocellular carcinoma. Int J Cancer. 2010;126:2067–78.
Zimmermann A, Saxena R. Hepatoblastoma. In: Bosman FT, Carneiro F, Hruban RH, et al., editors. WHO classification of tumours of the digestive system. Lyon: International Agency for Research on Cancer; 2010. p. 228–35.

Graft-Derived Cell-Free DNA as a Biomarker in Liver Transplantation

17

Michael Oellerich, Ekkehard Schütz, Julia Beck, Otto Kollmar, Philipp Kanzow, Anna Blum, and Philip D. Walson

Contents

M. Oellerich (✉) • P.D. Walson
Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany
e-mail: michael.oellerich@med.uni-goettingen.de; moeller@med.uni-goettingen.de; pwalson1@aol.com

E. Schütz • J. Beck
Chronix Biomedical GmbH, Göttingen, Germany
e-mail: esc@chronixbiomedical.de; jbeck@chronixbiomedical.de

O. Kollmar
Helios Dr. Horst Schmidt Kliniken Wiesbaden, Wiesbaden, Germany
e-mail: Otto.Kollmar@helios-kliniken.de

P. Kanzow
Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany

Department of Preventive Dentistry, Periodontology and Cariology, University Medical Center Göttingen, Göttingen, Germany
e-mail: philipp.kanzow@med.uni-goettingen.de

A. Blum
Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany
e-mail: anna.blum@med.uni-goettingen.de

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_10

Abstract

Improvement of long-term patient and graft outcome is still a challenge in liver transplantation. Personalized approaches to immunosuppressive treatment of liver transplant patients are currently under investigation, as conventional markers have limited usefulness to predict drug efficacy. The presence of graft-derived cell-free DNA (GcfDNA) in the plasma of liver transplant recipients opens up the possibility of monitoring allograft injury through measurement of this molecular marker. A rapid, cost-effective droplet digital PCR (ddPCR) method has been developed for the quantification of donor DNA. GcfDNA has shown to be useful for the detection of subclinical and full-blown acute rejection and non-rejection-related liver injury (e.g., HCV infection, liver trauma, ischemia/reperfusion damage). GcfDNA allows for the early detection of transplant injury ("liquid biopsy") and enables earlier more effective treatment intervention. It is especially helpful to guide changes in immunosuppression and to monitor immunosuppression minimization. This new approach may contribute to achieve more effective, less toxic personalized immunosuppression.

Keywords

Liquid biopsy • Transplant graft injury • Graft-derived cell-free DNA • Personalized immunosuppression • Acute rejection • Droplet digital PCR • Machine perfusion

List of Abbreviations

AST	Aspartate aminotransferase
cfDNA	Cell-free DNA
ddPCR	Droplet digital PCR
DNA	Deoxyribonucleic acid
GcfDNA	Graft-derived cell-free DNA
HCV	Hepatitis C virus
HELLP	Hemolysis, elevated liver enzymes, low platelets
ISD	Immunosuppressive drug
LFTs	Liver function tests
LTx	Liver transplantation
PCR	Polymerase chain reaction
TDM	Therapeutic drug monitoring
WBC	White blood cells
γ-GT	γ-glutamyltransferase

Key Facts About Personalized Immunosuppression

- The average half-life of transplanted livers is only about 10–13 years.
- Factors limiting long-term outcome in transplantation include irreversible chronic allograft dysfunction and adverse effects of standard immunosuppression, e.g., nephrotoxicity, cardiovascular disease, opportunistic infection, malignancy.
- New biomarkers are needed, as traditional approaches have serious limitations. TDM is more useful to prevent toxicity than to predict efficacy. Liver function tests are not diagnostic for assessing acute cellular rejection. Therefore, interventions may be too late.
- Organ transplants are also genome transplants and this opens up the possibility of monitoring for allograft injury through measurement of graft-derived cell-free DNA in plasma.
- Shortage of donor organs necessitates the use of more marginal donor organs. Ex vivo normothermic machine perfusion may promote graft acceptance. GcfDNA could provide useful information about effectiveness of organ preservation and graft integrity.
- Assessment of marginal grafts and improvement of outcome will be helpful to increase opportunities for patients on the waiting list to be transplanted despite a shortage of donor organs.
- Personalized immunosuppression will shift emphasis from reaction to prevention. It will make immunosuppressive therapy safer and reduce cost of health care.

Definitions of Words and Terms

Graft-derived cell-free DNA (GcfDNA)	GcfDNA is a cell death marker, released from necrotic or apoptotic cells of the transplanted organ into the systemic circulation. It can be determined in plasma.
Liquid biopsy	Liquid biopsy is a non-invasive approach to characterize and quantify circulating cell-free DNA in plasma either by sequencing or by quantitative digital PCR. The method has successfully been applied in transplantation, cancer, and autoimmune disease.
Machine perfusion	Ex-vivo normothermic machine perfusion is a tool to improve organ preservation in transplantation and to assess pre-transplant graft function.
Droplet digital PCR	This method allows a highly sensitive quantitative determination of DNA in plasma. The sample and reagents are partitioned into 20,000 droplets. DNA

	is randomly distributed among the droplets. The DNA is amplified for target detection using TacMan hydrolysis probes. After PCR amplification, each droplet provides a fluorescent positive or negative signal, indicating the target DNA was present or not present after partitioning. Each droplet provides an independent digital measurement. Positive and negative droplets are counted. A digital readout provides the amount of DNA.
Acute rejection	Under-immunosuppression can result in an acute rejection of the transplanted organ. Acute cellular rejection is mediated by effector T cells. This has to be distinguished from antibody-mediated rejection, e.g., by donor-specific HLA alloantibodies.
Ischemia/reperfusion damage	Ischemia/reperfusion damage is the result of oxidative stress and an inflammatory response during reperfusion of an ischemic organ.
Tolerance	Spontaneous operational tolerance is defined as long-term maintenance of stable graft function following discontinuation of conventional immunosuppression. This has to be distinguished from almost tolerance with stable graft function in minimally immunosuppressed recipients (low dose monotherapy). The estimated incidence of operational tolerance is $\leq$20% in liver transplantation, but with a much lower frequency in kidney transplantation.

Introduction

In 2013, about 30,000 patients in the USA received a new organ with another 80,000 patients on waiting lists, due to a shortage of donor organs. Every transplant graft that fails adds to the shortage of donor organs, and still today transplant patients often suffer from organ rejection. In liver transplantation (LTx), the rate of acute

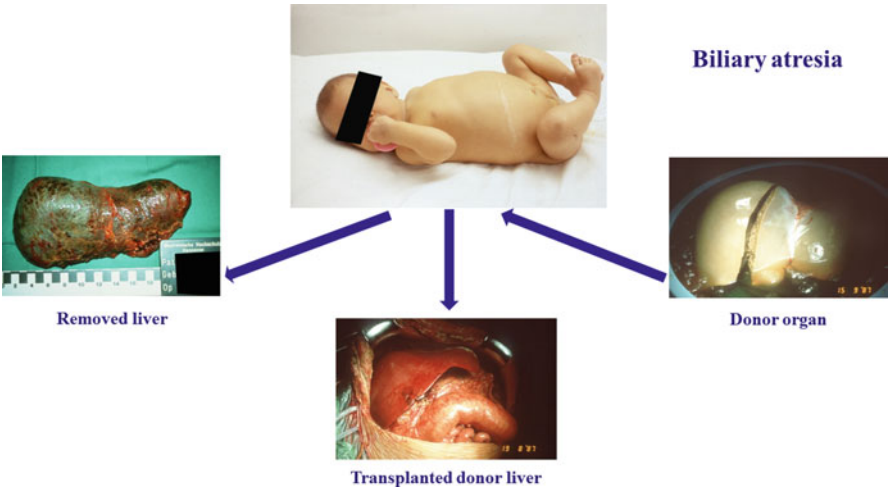

Fig. 1 Split liver transplantation in a pediatric patient with biliary atresia (Photo courtesy of Prof. Dr. Martin Burdelski, Medical School Hannover, 1987)

rejection at 3 years is about 22% (OPTN/SRTR 2010 Annual Data Report; OPTN/SRTR 2013 Annual Data Report).

Long-term patient and graft outcomes are a particular challenge in pediatric liver transplantation. As an example, Fig. 1 shows a less than 1-year-old child with biliary atresia who had a previous, unsuccessful Kasai operation and received a left lateral liver segment from a deceased donor. The average pediatric liver transplant patient survival at 20 years is only 50–64% (Gallo and Esquivel 2013) with graft failure rate within 10 years in pediatric patients of 40% (Yazigi 2013).

The limited graft and patient survivals are the result of two major factors, namely, irreversible chronic allograft dysfunction and the adverse effects of standard immunosuppression, such as nephrotoxicity, cardiovascular disease, opportunistic infection, and malignancy.

There are numerous causes of chronic liver allograft dysfunction. Immunological factors include previous sensitization, episodes of acute rejection, subacute or chronic alloimmune responses, suboptimal immunosuppression, and noncompliance of patients. Other factors include disease recurrence and non-immunological factors, such as marginal graft quality, preservation or ischemic injury, acute peritransplant injuries, hypertension, hyperlipidemia, and chronic toxic effects of cyclosporine or tacrolimus (Pascual et al. 2002).

Personalized Immunosuppression

As in other areas of medicine, personalized approaches to the treatment of transplant patients are rapidly becoming an essential component of patient care. In order to overcome the traditional limitations of "trial and error" health-care practices and

protocol-based approaches, individualized treatments, tailored to each patient, are being used. Therapeutic drug monitoring (TDM) is the accepted procedure to personalize immunosuppressant drug (ISD) dosage, but the ability of TDM to prolong graft or patient survival is limited. It is more useful to prevent toxicity than to predict ISD efficacy (Crettol et al. 2008; Falck et al. 2008). Conventional markers of liver damage [e.g., liver function tests (LFTs)] are also used to adjust therapy but have limited usefulness because they are not diagnostic for assessing acute cellular rejection (Rodríguez-Perálvarez et al. 2012, 2013). LFTs therefore are inadequate to effectively guide timely intervention. For these reasons, there is a need for new biomarkers that can be used to personalize the clinical ISD management of organ recipients. There is a special need for biomarkers that detect subclinical as well as acute rejection and to assess minimal necessary ISD exposure to guide therapeutic decision-making. Such biomarkers should be capable of identifying silent graft injury since this can lead to acute rejection or chronic allograft dysfunction. To be clinically useful, such biomarkers should be practical with a short turnaround time (same day), affordable, and minimally invasive.

Graft-Derived Circulating Cell-Free DNA as a Marker of Liver Injury

Development of Molecular Methods

The presence of donor-specific DNA in the plasma of kidney and liver transplant recipients was described more than a decade ago (Lo et al. 1998). Using PCR, this group detected ChrY specific cell-free DNA in female recipients of organs from male donors, showing that cell-free donor DNA was in the circulation. It was proposed to be a cell death marker, released from necrotic or apoptotic cells of the transplanted organ into the bloodstream of the recipient, and therefore might be useful as a biomarker for allograft rejection. This approach took advantage of the fact that organ transplants are also genome transplants with the possibility of monitoring for allograft injury through measurement of graft-derived cell-free DNA (GcfDNA) in the plasma for any allograft.

More recently, this concept was applied in a study that used urinary cell-free DNA (cfDNA) as a surrogate marker of kidney transplant injury (Sigdel et al. 2013). Urine samples from 63 female recipients of grafts from male donors (41 stable, 22 allograft injury) were analyzed for ChrY (donor-specific) cfDNA in this study. It was shown that in patients with acute rejection, significantly higher values could be detected, than in patients with a stable graft or in those suffering from chronic allograft injury. There was, however, no significant difference in cfDNA values between patients with acute rejection and BK virus nephropathy. However, the most serious limitation of this approach of measuring ChrY donor-derived cell-free DNA is that it can only be applied to a limited number of allograft recipients, since sex mismatch pairing (male graft to female recipient) is required.

Snyder et al. developed a method that could be used in the absence of this specific gender pairing that is based on shotgun sequencing (Snyder et al. 2011). For this approach, both donor and recipients need to be subjected to single nucleotide polymorphism (SNP) typing with, e.g., SNP microarray techniques to interrogate sentinel SNPs. After isolation of cell-free DNA from the plasma, shotgun sequencing was performed to count reads identified to be donor and recipient specific, based on the SNP alleles in order to calculate the percentage of donor DNA in the cell-free DNA. The donor DNA percentage was then monitored over time to detect the onset of rejection in heart transplant recipients.

For a test to be useful for therapeutic decision-making in a clinical setting, test results must be available in a timely fashion and, particularly in situations such as acute rejection, within the same day. While costs continue to decrease, sequencing and the bioinformatics interrogation of the SNP alleles, such as done by Snyder et al., are still both relatively time consuming and expensive. To overcome these disadvantages, a universal droplet digital PCR (ddPCR) method was established for the first time to rapidly quantify GcfDNA percentages in the circulation of liver transplant recipients (Beck et al. 2013). This new ddPCR test can be used as a universal biomarker of graft health or injury. It is both cost-effective and rapid, providing results within the same day. The current status of using this promising biomarker in transplantation has recently been reviewed (Gielis et al. 2015).

Method Description: Use of Droplet Digital PCR for the Assessment of Percent GcfDNA and Absolute Quantification

The first step is to obtain a blood sample from the graft recipient. To avoid release of DNA from the white blood cells (WBC), which occurs if whole blood is stored for more than 6 h, special blood collection tubes, which stabilize WBC (e.g., Cell-Free DNA BCT Tubes, Streck Inc, Omaha, USA), can be used. The latter allows for shipment of drawn blood for analysis, without the need of on-site centrifugation and plasma collection. The next step is to isolate cell-free DNA from the plasma and to harvest white blood cells, which are used to identify recipient-specific homozygous SNPs. A further step is to identify informative assays from a library of about 40 preselected SNPs with a population minor allelic frequency >40%. Useful SNPs are those which are homozygous in recipient and in graft, but having different alleles. On average, five such heterologous SNP loci are obtained and used for analysis by digital PCR. The third step is to perform droplet digital PCR assay on these selected SNP loci and quantify target nucleic acids to calculate percent of GcfDNA (Beck et al. 2013). In the droplet digital PCR system used (QX200, Bio-Rad, Pleasanton, USA), the reagents and diluted samples are partitioned into 20,000 droplets. After PCR amplification, using TaqMan hydrolysis probes with different fluorophores for the alleles, each droplet provides a fluorescent positive or negative signal indicating a target DNA molecule was present or absent. Positive and negative droplets are counted. Each droplet provides an independent digital

measurement of the target molecules that were present in the extracted cfDNA. The digital readout is then converted into a percentage of circulating graft-derived cell-free DNA (GcfDNA).

The fact that any variability in the amount of recipient cfDNA may mask an increase of graft DNA when the cfDNA percentage is used prompted the development of a method for absolute quantification of GcfDNA (Beck et al. 2014). This new droplet digital PCR uses synthetic nonhuman DNA as an internal standard. GcfDNA concentration is calculated by multiplying the total amount of cfDNA (determined using the internal standard) by the percentage of GcfDNA defined as shown in the formula below:

$$\text{GcfDNA (cp/mL)} = [\text{total cfDNA (cp/mL)}] \times [\text{GcfDNA (\%)}]$$

While the absolute amount of GcfDNA has some theoretical advantages over the GcfDNA percentage, the actual superiority of this approach is currently still under investigation (Beck et al. 2014).

Clinical Applications

There are numerous clinical conditions where a biomarker of liver graft integrity would be desirable. Such settings include the assessment of ischemia/reperfusion damage, detection of subclinical as well as full-blown rejection, verification of clinically suspected acute rejection, identifying non-rejection-related injury, and guidance of immunosuppression especially during attempts at ISD minimization and clinically indicated switching of immunosuppressants.

Due to the shortage of donor organs, it has become necessary to make use of more marginal donor organs. This resulted in the development of a new portable ex vivo normothermic organ perfusion device as an alternative to cold storage. Machine perfusion seems to be useful for the assessment of pretransplant graft function during normothermic preservation (Van Raemdonck et al. 2013). This procedure may also promote graft acceptance allowing for downregulation of adaptive immunity. GcfDNA could in future be a valuable tool to assess the effectiveness of this procedure regarding preservation of graft integrity. With conventional cold organ preservation, a very high increase in GcfDNA is observed in the recipient on the first day after transplantation due to ischemia/reperfusion damage, which will probably be much less expressed using this new machine perfusion technique. Therefore monitoring of GcfDNA during machine perfusion and after graft implantation could provide useful information about organ preservation. It could be shown that the amount and dynamics of GcfDNA release during the first week after engraftment can serve to assess the extent of ischemia/reperfusion damage (Beck et al. 2015). In living-related kidney transplantation, a significantly lower area under the curve ($AUC_{\text{day } 0-5}$) of GcfDNA was observed, compared to deceased donor organ transplantation.

The following single case illustrates the relevance of the clinical application of GcfDNA (Fig. 2). This (previously reported, Kanzow et al. 2014) patient had

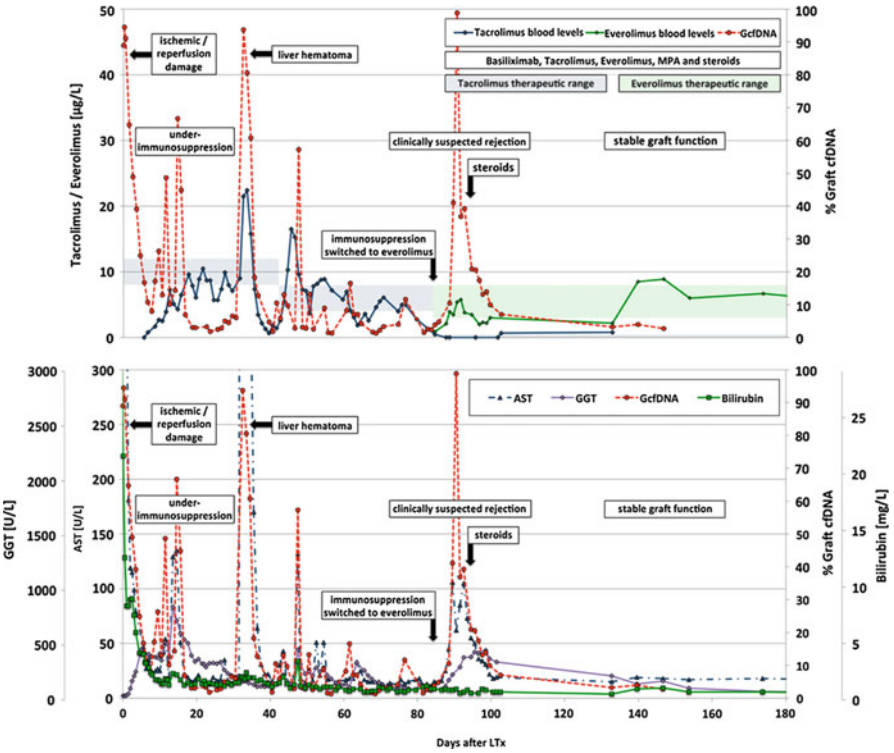

Fig. 2 Graft-derived cell-free DNA (GcfDNA) values and tacrolimus/everolimus trough blood levels in a recipient of a marginal HELLP syndrome donor liver. The *lower panel* shows serum AST, bilirubin, and γ-GT values (Kanzow et al. 2014. Reproduced with permission of Wolters Kluwer Health, Inc.)

received a marginal liver graft from a donor who died from HELLP syndrome. There was a rapid decline from the highly elevated initial GcfDNA values due to ischemia/reperfusion damage that fell below the threshold of 10% GcfDNA on day 8. The dynamics of this decrease was in the range usually seen with non-marginal donor grafts. During the first 19 days post LTx, because the patient was on intermittent dialysis, tacrolimus concentrations were kept below the therapeutic range. This resulted in repeated elevations of GcfDNA, indicating graft damage that eventually stabilized. However, on day 19 the patient fell and had liver trauma resulting in a large hematoma accompanied by a marked increase of GcfDNA, caused by this non-rejection-related graft injury. At this time, tacrolimus concentrations were very high despite no change in dosage: most likely the result of decreased hepatic metabolism of tacrolimus. In response to the elevated tacrolimus concentrations, the dosage was reduced, resulting in under-immunosuppression that again resulted in an increase of GcfDNA. Finally, the patient was switched from tacrolimus to everolimus. During the switch, over a short period of insufficient immunosuppression, the patient developed acute rejection that was reflected

Table 1 GcfDNA early (days 8–30) after liver transplantation

Patients	N[b]	n[c]	GcfDNA (%)
Stable, HCV-, Tacro ≥8 μg/L	16	75	4.3 (1.1–9.6)[d]
Stable*, HCV-, Tacro <8 μg/L	15	122	6.9 (1.2–30.4)[d]
Stable*, HCV+, Tacro ≥8 μg/L	6	28	11.6 (2.8–26.5)[d]
Stable, HCV+, Tacro <8 μg/L	6	34	9.2 (1.2–35.1)[d]
Cholestasis, no rejection[a]	5	41	3.3 (1.6–5,9)[d]
4–6 days before rejection*[a]	4	7	20.7 (10.5–54.1)[d]
Acute rejection*[a]	6	20	33.2 (10.8–70.3)[d]

Adapted data from Oellerich et al. 2014a. Reproduced with permission of John Wiley and Sons
*$p \leq 0.0005$ compared to stable patients (HCV-, Tacro ≥8 μg/L) by Wilcoxon test
[a]Includes samples beyond 30 days
[b]Number of patients
[c]Number of contributing values
[d]Median (5–95th percentile)

by a pronounced increase of GcfDNA. This rejection episode responded to steroid bolus treatment, as indicated by a rapid decrease of GcfDNA. Then the patient stabilized after everolimus was optimized and the patient was still doing well for more than a year posttransplant.

The diagnostic usefulness of GcfDNA is summarized in Table 1. Characteristic changes of GcfDNA were associated with various clinical conditions in a subset of LTx patients from an ongoing trial, who were receiving ISD regimens with tacrolimus, mycophenolic acid, and steroids. Stable HCV-negative patients with tacrolimus levels within the therapeutic range showed GcfDNA values below 10%, indicating no graft damage. In stable patients with subtherapeutic tacrolimus levels early after transplantation, 9/15 had elevated GcfDNA values, indicating organ injury. The majority of patients who were HCV positive had in general elevated GcfDNA values, which seems to reflect infection-related liver damage. Patients with acute rejection had highly elevated GcfDNA values, compared to stable patients ($p < 0.0001$). Noteworthy GcfDNA increases were observed as early as 4–6 days before the acute rejection was clinically apparent or suspected. In five patients with either intrahepatic or drug-induced cholestasis or post-liver transplant cholangiopathy who showed no clinical signs of rejection, there was no elevation of GcfDNA, in contrast to conventional markers like aspartate aminotransferase (AST) and γ-glutamyltransferase (γ-GT), indicating a higher specificity of the cfDNA test for hepatocellular damage.

Identification of the minimally effective immunosuppressive drug concentrations needed in an individual patient is another important clinical challenge that is not accomplished by drug monitoring. Figure 3 shows GcfDNA versus tacrolimus trough levels during days 5–30 postsurgery. In patients with subtherapeutic tacrolimus trough concentrations (<7 ng/mL), most of the GcfDNA values were substantially elevated above the threshold of 10%, indicating graft injury (Oellerich et al. 2014b). However, there were patients with elevated as well as low GcfDNA in the tacrolimus level range

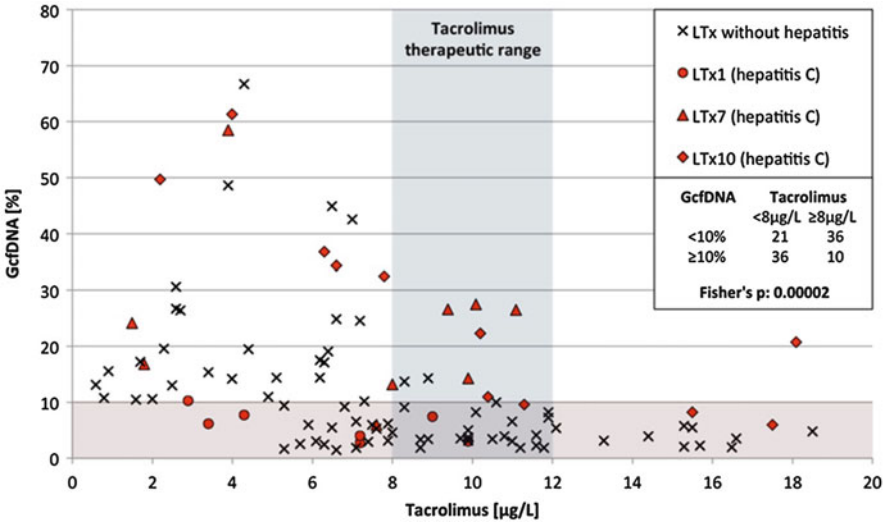

Fig. 3 Comparison of the percentages of graft-derived cell-free DNA (GcfDNA) versus tacrolimus trough blood levels in adult liver transplant recipients ($n = 10$) during the first 5–30 days after transplantation (Oellerich et al. 2014b. Reproduced with permission of Wolters Kluwer Health, Inc.)

between 6 and 9 ng/mL, indicating the individual differences in ISD exposure needed to control immune response, which is not reflected by ISD monitoring. GcfDNA could therefore be helpful to guide immunosuppression minimization.

In an independent LTx study (Rodríguez-Perálvarez et al. 2013), it was recently demonstrated that graft loss over 10 years was significantly higher in patients with tacrolimus concentrations <7 ng/mL early after transplantation. In such patients, graft loss was twice as high as in patients with tacrolimus concentrations between 7 and 15 ng/mL. Long-term studies will demonstrate whether the graft-derived cell-free DNA in combination with ISD monitoring will be a superior (e.g., more precise) predictor of long-term outcome. The method has now been used in over 120 LTx patients as part of a prospective multicenter trial.

For biomarker-guided minimization trials, GcfDNA monitoring could therefore be useful. In LTx, the estimated incidence of operational tolerance is ≤20% (Londoño et al. 2012). During minimization it is expected that GcfDNA would increase in patients who need stronger immunosuppression. In such patients, effective CNI doses could be restored in time to avoid acute rejection.

Limitations

GcfDNA detects organ injury from both acute rejection and non-rejection-related events (e.g., HCV infection, liver trauma, or liver ischemia). Other factors that may influence GcfDNA values may include delayed graft function, drug toxicity, or tumors. The effect of over-immunosuppression has not been formally studied, but

to date we have not seen GcfDNA elevations with ISD concentrations above the therapeutic range (Fig. 3). For these reasons, GcfDNA results have to be evaluated in context with all available clinical findings (Oellerich et al. 2015).

The determination of GcfDNA requires specific expertise and specialized instrumentation (ddPCR).

Conclusion

GcfDNA is a very useful complement to TDM for posttransplant monitoring, enabling a direct real-time assessment of graft integrity. It is a cost-effective approach with same day turnaround, when performed in a molecular diagnostic laboratory. In contrast to conventional liver function tests, GcfDNA allows for the early detection of transplant injury ("liquid biopsy") and enables earlier, more effective treatment intervention.

It may be most useful in situations where TDM is inadequate, such as during combination therapy, ISD switches, and to identify compliance problems. It may also be especially helpful to guide changes in immunosuppression and to monitor immunosuppression minimization. The ability to detect silent subclinical liver damage is important in avoiding the development of chronic allograft dysfunction. This new approach may contribute to achieving more effective, less toxic personalized immunosuppression in liver transplantation. Effective, truly personalized immunosuppression would shift emphasis from reaction to prevention. GcfDNA monitoring will provide actionable health-care information, allowing for the right therapy for the right patient. It will improve immunosuppressive therapy, which is of great benefit for LTx patients, and could reduce the cost of health care.

Summary Points

- This chapter focuses on graft-derived cell-free DNA (GcfDNA) in plasma as a marker of graft injury ("liquid biopsy"). A rapid, cost-effective droplet digital PCR method is available for the quantification of GcfDNA in plasma.
- GcfDNA is useful for the detection of subclinical and full-blown acute rejection and non-rejection related liver injury.
- It is useful for verification of clinically suspected acute rejection.
- It may be helpful to guide changes in immunosuppression and to assess minimal necessary exposure. GcfDNA could be helpful to early detect silent graft injury leading to acute rejection or chronic allograft dysfunction.
- This new approach may contribute to more effective personalized immunosuppression in transplantation.

References

Beck J, Bierau S, Balzer S, et al. Digital droplet PCR for rapid quantification of donor DNA in the circulation of transplant recipients as a potential universal biomarker of graft injury. Clin Chem. 2013;59:1732–41.

Beck J, Schmitz J, Kanzow P, et al. Absolute quantification of graft derived cell-free DNA (GcfDNA) early after liver transplantation (LTx) using droplet digital PCR. Clin Chem. 2014;60(Suppl):S194–5.

Beck J, Oellerich M, Schulz U, et al. Donor derived cell-free DNA is a novel universal biomarker for allograft rejection in solid organ transplantation. Transplant Proc. 2015; 47:2400–3.

Crettol S, Venetz JP, Fontana M, et al. Influence of *ABCB1* genetic polymorphisms on cylcosporine intracellular concentration in transplant recipients. Pharmacogenet Genomics. 2008;18:307–15.

Falck P, Åsberg A, Guldseth H, et al. Declining intracellular T-lymphocyte concentration of cyclosporine a precedes acute rejection in kidney transplant recipients. Transplantation. 2008;85:179–84.

Gallo A, Esquivel CO. Current options for management of biliary atresia. Pediatr Transplant. 2013;17:95–8.

Gielis EM, Ledeganck KJ, De Winter BY, et al. Cell-free DNA: an upcoming biomarker in transplantation. Am J Transplant. 2015;15:2541–51.

Kanzow P, Kollmar O, Schütz E, et al. Graft-derived cell-free DNA as an early organ integrity biomarker after transplantation of a marginal HELLP syndrome donor liver. Transplantation. 2014;98:e43–5.

Lo YMD, Tein MSC, Pang CCP, et al. Presence of donor-specific DNA in plasma of kidney and liver-transplant recipients. Lancet. 1998;351:1329–30.

Londoño MC, Danger R, Giral M, et al. A need for biomarkers of operational tolerance in liver and kidney transplantation. Am J Transplant. 2012;12:1370–7.

Oellerich M, Kanzow P, Beck J, et al. Graft-derived cell-free DNA (GcfDNA) as a sensitive measure of individual graft integrity after liver transplantation. Am J Transplant. 2014a;14 (Suppl3):874.

Oellerich M, Schütz E, Kanzow P, et al. Use of graft-derived cell-free DNA as an organ integrity biomarker to reexamine effective tacrolimus trough concentrations after liver transplantation. Ther Drug Monit. 2014b;36:136–40.

Oellerich M, Walson PD, Beck J, et al. Graft-derived cell-free DNA as a marker of transplant graft injury. Ther Drug Monit. 2016; 38(Suppl 1):575–9.

Organ Procurement and Transplantation Network (OPTN) and Scientific Registry of Transplant Recipients (SRTR). OPTN/SRTR 2010; annual data report. Rockville: Department of Health and Human Services, Health Resources and Services Administration, Healthcare Systems Bureau, Division of Transplantation; 2011.

Organ Procurement and Transplantation Network (OPTN) and Scientific Registry of Transplant Recipients (SRTR). OPTN/SRTR 2013: annual data report. Am J Transplant. 2015;15(Issue S2):4–13.

Pascual M, Theruvath T, Kawai T, et al. Strategies to improve long-term outcomes after renal transplantation. N Engl J Med. 2002;346:580–90.

Rodríguez-Perálvarez M, Germani G, Darius T, et al. Tacrolimus trough levels, rejection and renal impairment in liver transplantation: a systematic review and meta-analysis. Am J Transplant. 2012;12:2797–814.

Rodríguez-Perálvarez M, Germani G, Papastergiou V, et al. Early tacrolimus exposure after liver transplantation: relationship with moderate/severe acute rejection and long-term outcome. J Hepatol. 2013;58:262–70.

Sigdel TK, Vitalone MJ, Tran TQ, et al. A rapid noninvasive assay for the detection of renal transplant injury. Transplantation. 2013;96:97–101.

Snyder TM, Khush KK, Valantine HA, et al. Universal noninvasive detection of solid organ transplant rejection. Proc Natl Acad Sci U S A. 2011;108:6229–34.
Van Raemdonck D, Neyrinck A, Rega F, et al. Machine perfusion in organ transplantation: a tool for ex-vivo graft conditioning with mesenchymal stem cells? Curr Opin Org Transplant. 2013;18:24–33.
Yazigi NA. Long term outcomes after pediatric liver transplantation. Pediatr Gastroenterol Hepatol Nutr. 2013;16:207–18.

Cortisol as Biomarkers in Cirrhosis

18

Luisa Spadaro, Graziella Privitera, Giuseppe Fede, Giovanni Meli, and Francesco Purrello

Contents

Abstract

Cortisol is the main glucocorticoid in humans. It is released in a dynamic and circadian manner from the adrenal cortex. Its secretion is regulated by the Hypothalamus-pituitary-adrenal axis. Glucocorticoids function to maintain homeostasis both in response to normal diurnal changes in metabolism and in the face of stressful perturbations. The physiological actions of glucocorticoids are mediated by the glucocorticoid receptor that is expressed in nearly every cell of the body. Increasing evidences support the role of cortisol as biomarker of liver disease. An impairment of adrenal function is a newly defined complication of liver cirrhosis. The term hepato-adrenal syndrome was introduced to define the presence of adrenal insufficiency in patients with advanced liver disease with or

L. Spadaro (✉) • G. Privitera (✉) • G. Fede (✉) • G. Meli (✉) • F. Purrello (✉)
Department of Clinical and Experimental Medicine, University of Catania- Garibaldi Hospital, Catania, Italy
e-mail: luspa70@hotmail.it; luspa70@hotmail.com; graziella.privi@gmail.com; g_fede@tiscali.it; dottmeli@hotmail.it; fpurrell@unict.it

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_39

without sepsis. The prevalence of adrenal insufficiency is strictly related to disease severity in term of Child Pugh and model for end-stage liver disease scores. The diagnosis of adrenal insufficiency in cirrhosis constitutes a crucial point. Published studies have used a variety of biochemical criteria to define abnormalities in adrenal function during liver cirrhosis. Common methods used in the general population to assess adrenal insufficiency could be invalid in cirrhotic patients because there are a number of confounding factors that make interpretation difficult. The common methods for assessing adrenal function, based on total cortisol, may lead to overestimation of adrenal insufficiency in patients with cirrhosis. The optimal method could be the direct evaluation of free cortisol, but its measurement is difficult in daily clinical practice. The determination of serum free cortisol provides not only the best estimation of adrenal function but has also a prognostic value. Salivary cortisol is a good surrogate marker of free cortisol, but its assay need to be standardized. Cortisol response in cirrhotic patients is an independent prognostic factor. In septic patients, in variceal bleeding, and also in noncritically cirrhotic patients, adrenal insufficiency is related with worse prognosis. Cortisol is a surrogate marker of inflammatory stress, reflecting systemic inflammatory response to intestinal bacterial translocation and occult infections. Adrenal dysfunction may contribute to cardiovascular derangement and represents a marker of circulatory dysfunction. In long-standing decompensated cirrhosis, adrenal glands respond poorly to hypotensive stress, preventing a rise in cortisolemia and reducing the vascular effects of vasoconstrictors. Finally, reduced release of corticosterone from adrenal glands contribute to the impairment of circadian rhythms of motor activity in hyperammonemia and hepatic encephalopathy.

Keywords

Adrenal insufficiency • Cirrhosis • Cortisol • Hepato-adrenal syndrome • Hypothalamus- pituitary- adrenal axis

List of Abbreviations

11β-HSD-1	11β-hydroxysteroid dehydrogenase-1
11β-HSD-2	11β-hydroxysteroid dehydrogenase-2
ACTH	Adrenocorticotropic hormone
AI	Adrenal insufficiency
AP-1	Activator protein-1
CBG	Corticosteroid binding globulin
CNS	Central nervous system
CRH	Corticotropin- releasing hormone
CRP	C-reactive protein
DBD	Central-DNA binding domain
EEG	Electroencephalograms
FCI	Free cortisol index
GCs	Glucocorticoids
GEVB	Gastroesophageal variceal bleeding

GI	Gastrointestinal
GR	Glucocorticoid receptor
HDL	High-density lipoprotein
HE	Hepatic encephalopathy
HPA	Hypothalamus-pituitary-adrenal
HRS	Hepatorenal syndrome
Hsp	Heat shock protein
LBD	C-terminal ligand-binding domain
LD-SST	Low dose short synacthen test
MELD	Model for end-stage liver disease
NAF	Normal adrenal function
NAFLD	Nonalcoholic fatty liver disease
NF-kB	Nuclear factor- kB
NTD	N-terminal transactivation domain
RAI	Relative adrenal insufficiency
REM	Rapid eye movement
SD-SST	Standard dose short synacthen test
SFC	Serum free cortisol
Smad	Sma and Mad- related proteins
STAT	Signal transduction and activator of transcription
SWS	Slow wave sleep
T2D	Type-2 diabetes

Key Facts

- Cortisol is the main glucocorticoid hormone in humans and is produced by the adrenal glands, which are small glands located above the kidneys. Synthesis and release of cortisol is under dynamic circadian and ultradian regulation by other two glands: hypothalamus and pituitary gland, located in the brain. These three glands are interconnected and form the Hypothalamic-pituitary-adrenal (HPA) axis.
- Cortisol is released in response to stress and low blood-glucose concentration and has several roles which are crucial during stress condition: it increases production and mobilization of glucose from other organ (muscles, liver); it prevents an over activation of the inflammatory response during infection or injury; it has hemodynamic effect that contributes to maintain efficient blood pressure during stress condition.
- Adrenal insufficiency is defined as deficient production or action of cortisol resulting from either a structural damage of adrenal glands, "primary adrenal failure," or an impairment of the hypothalamic-pituitary axis, "secondary adrenal failure".
- Adrenal insufficiency is a disorder characterized by the failure of the production and/or action of cortisol. It can be primary or secondary. Primary AI occurs when

there is a structural damage of the adrenal glands, in contrast, secondary AI occurs when there is an impairment of the HPA axis.
- Adrenal insufficiency has been documented in patients with chronic liver disease. It usually is asymptomatic, but during stress condition may worsen the prognosis of these patients, causing shock, kidney failure, sepsis.

Definition of Words and Terms

ACTH stimulation test	This test represents the gold standard for the diagnosis of adrenal insufficiency. Whatever the cause, the diagnosis of AI depends entirely on the demonstration that cortisol secretion is inappropriately low. The test entails the stimulation of the adrenal glands by pharmacological doses (250 or 1 μg) of exogenous corticotropin 1–24 which has the full biological potency of native 39-aminoacid corticotropin.
Cortisol binding globulin (CBG)	It is the principal transport protein of glucocorticoids. CBG is mainly produced by hepatocytes in the liver. It plays a vital role as a circulating reservoir of cortisol in the circulation. CBG influences and modulates the bioavailability of cortisol to local tissues. The concentration of free cortisol depends primarily on the binding activity and the concentration of CBG. Functions and synthesis of CBG vary with genetic factors, gender, temperature, glycosylation state, and concomitant medications.
Free cortisol	Free cortisol is the unbound component of secreted cortisol. It represents the smallest part (10% of total cortisol) but is the most important because only free cortisol can bind to its receptor to elicit physiological effects and is thus biologically active. Free cortisol is not always proportional to total cortisol such as hypercortisolemia or hypoproteinemia, in these conditions the direct measurement of free cortisol is mandatory.
HPA axis	The hypothalamus-pituitary-adrenal axis is a complex set of relationships and signals

	that exist between the hypothalamus, the pituitary gland, and the adrenals. The HPA axis is an ever-dynamic intertwining of the central nervous system and endocrine system. It is responsible for the adaptation component of the stress response. In response to a stressor, the hypothalamus releases CRH which triggers the release of ACTH from the pituitary gland. ACTH travels through the bloodstream and stimulates the adrenal glands to release glucocorticoids.
Relative adrenal insufficiency (RAI)	It is generally accepted that a pronounced activation of the HPA axis in response to critical illness is vital. In this setting, the term of RAI has been used to define a transient and functional deficiency of cortisol production related to the disease severity. During critical illness, the degree of HPA axis activation could be not enough to cover the increased need of cortisol to survive, even if absolute plasma cortisol levels are very high.

Introduction

Cortisol is the principal glucocorticoid in humans and circulating levels are controlled by hypothalamus- pituitary- adrenal (HPA) axis. Synthesis and release of glucocorticoids (GCs) is under dynamic circadian and ultradian regulation by the periventricular nucleus of the hypothalamus. Corticotropin- releasing hormone (CRH) secreted by the hypothalamus stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. In turn, ACTH induces the synthesis and secretion of cortisol from the cortex of the adrenal glands into the bloodstream (Fig. 1). The adrenal cortex has three distinct zones, which secrete the various hormones. Cortisol secretion from the zona fasciculata is primarily regulated by corticotropin (ACTH) and arginine vasopressin. In healthy people, cortisol secretion is pulsatile, and circulating cortisol concentrations fluctuate naturally in a circadian fashion, highest in the early morning (0600–0800 h) and lowest around midnight. Glucocorticoids from the adrenal cortex have several roles which are crucial at times of infection or injury when they promote resolution of the inflammatory response, mobilize fuel, and allow hemodynamic adaptation to stress (Biddie et al. 2012; Ramamoorthy and Cidlowski 2013). Approximately 80% of circulating cortisol is synthesized both at rest and during stress from plasma cholesterol (particularly in the form of HDL cholesterol), and this is relevant in patients

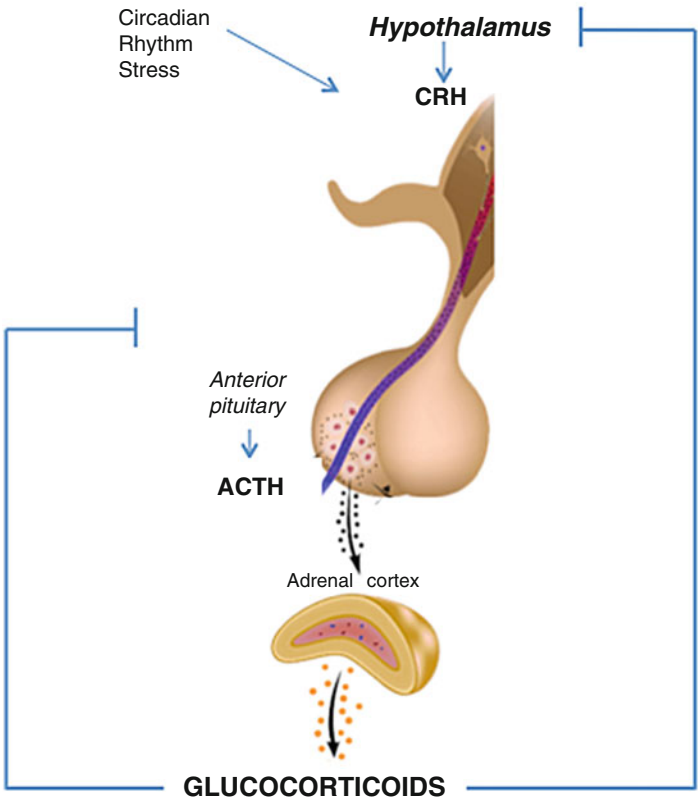

Fig. 1 Regulation of glucocorticoid hormone secretion by the hypothalamic-pituitary- adrenal (*HPA*) axis

with liver cirrhosis where cholesterol is low and may limit the synthesis of cortisol (Cicognani et al. 1997; Spadaro et al. 2015).

Tissue cortisol concentrations are controlled by a series of enzymes that regenerate and deactivate GCs (tissue GCs metabolism). These include 11β-hydroxysteroid dehydrogenases (11β-HSD1 + 11β-HSD2) and A-ringreductases. 11β-HSD1 is most highly expressed in liver, adipose tissue, lung, and areas of central nervous system (CNS). In these sites, it is associated with glucocorticoid, not mineralcorticoid, receptors. In vivo it functions as a predominant reductase converting cortisone to cortisol. Because glucocorticoid receptors have relatively low affinity for cortisol, and cortisone levels in blood are relatively constant; the physiologic role of 11β-HSD1 is to maintain adequate activation of glucocorticoid receptors in sites where this is vital to metabolic functions. 11β-hydroxysteroid dehydrogenase type 2 catalyzes the inactivation of cortisol to inert cortisone. It is expressed in distal nephron and few other sites, where its presence is required in order to prevent cortisol from gaining access to mineralcorticoid receptors (Walker and Andrew 2006). The liver itself has been shown to produce significant amounts of

cortisol into the splanchnic circulation. Glucocorticoids are thought to diffuse freely across the cell membrane because of their lipophilicity. The effects of GCs are mediated via the glucocorticoid receptor (GR), which is a member of the steroid hormone receptor superfamily. Once in the cytoplasm, they interact with the GR that mediates most of the hormone-induced actions. The GR comprises three major functional domains, a N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), and C-terminal ligand-binding domain (LBD). Recent studies revealed that alternative splicing of the human GR transcript produces multiple isoforms. These appear to play a role in regulating cellular sensitivity to GCs. GR-α is the active form of glucocorticoid receptor. The alternative splicing of exon 9 produces the inactive β isoform; increased expression of the GR β isoform, cause relative glucocorticoid resistance. The two isoforms are identical proteins up through amino acid 727 corresponding to the C-terminal end of helix 10 of the LBD, but the remainder of the ligand-binding domain differs between the two receptors. hGR α has an additional 50 amino acids that encode helices 11 and 12 to complete a functional domain capable of binding ligand and activating gene expression. In contrast, hGR β is unable to bind ligand and to directly activate glucocorticoid responsive promoters (Kadmiel and Cidlowski 2013; Oakley and Cidlowski 2013).

GR α primarily resides in the cytoplasm as part of a multisubunit complex, including Hsp90, Hsp70, Hsp40, immunophilins, cyclophilin 40 (CyP40), and P23 protein. In response to GCs, the GR α complex rapidly undergoes a conformational change and subsequently dissociated from the heat shock proteins. Therefore GR is able to produce rapid non-genomic actions through interactions with signaling pathways via cytosolic kinases and to translocate into the nucleus. The interaction of GR with the proinflammatory transcription factors activator protein 1 (AP1) and nuclear factor kB (NF-kB) antagonizes their activity and is considered to be a primary mechanism through which glucocorticoids suppress inflammation. Into the nucleus, activated GR binds to consensus elements in the host cell genome to activate or repress gene transcription. GR interacts with both the DNA elements and other transcription factors such as NF-kB, AP-1, Sma, and Mad-related protein (Smad), and signal transduction and activator of transcription (STAT) (Zhou and Cidlowski 2005).

Cortisol as Marker of Hepato-Adrenal Syndrome in Liver Cirrhosis

There is an increasing body of literature regarding adrenal function in liver cirrhosis (Fig. 2).

Nowadays liver cirrhosis is considered to be among the major groups of high-risk diseases with a predisposition to develop adrenal insufficiency (Bornstein 2009). The term hepato-adrenal syndrome is used to define the presence of adrenal insufficiency (AI) in patients with advanced liver disease, suggesting that adrenocortical insufficiency is a feature of liver disease per se, with a different pathogenesis from that occurring in septic shock. Up to now, the diagnosis of AI is based on laboratory tests because the clinical characteristics of cirrhotic patients with hepato-adrenal

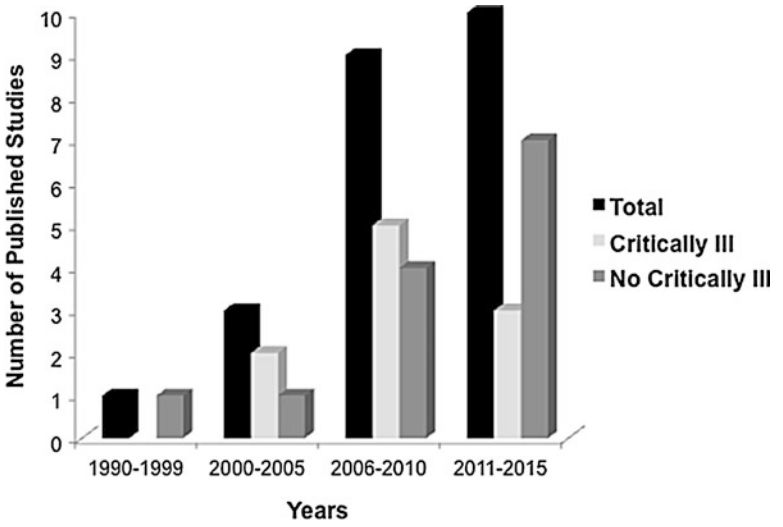

Fig. 2 Number of studies published per year regarding liver cirrhosis and adrenal function. The year 2016 was excluded from the graph as the complete results for this year are not yet available

syndrome are unknown and difficult to define. Measurement of serum total cortisol, either at baseline or following stimulation by the synacthen test is the gold standard for the diagnosis of AI in liver cirrhosis. AI is generally diagnosed by the ACTH stimulation test, which is safe and reliable. Other tests assessing the integrity of the entire HPA axis have been used. Such tests include the insulin-induced hypoglycaemia, metyrapone testing and CRH test. All of these tests are unsafe and impractical, and the data are difficult to interpret in the setting of liver disease. Baseline serum total cortisol levels under 414 nmol/L, <250 nmol/L, or <138 nmol/L have been used to AI in different studies (Trifan et al. 2013). Furthermore, there are two different stimulation tests to evaluate the adrenal reserve in this setting: the standard dose short synacthen test (SD-SST) and the low-dose short synacthen test (LD-SST). SD-SST measures total serum cortisol at baseline and 60 min after an intravenous injection of 250 μg of synthetic ACTH. SD-SST is not a "physiological test" because the dose used is approximately 100 times higher than normal maximal stress ACTH levels, thus, using a supraphysiological dose of corticotrophin, SD-SST is preferentially utilized in critically ill cirrhotic patients. Although there is no consensus on the diagnostic criteria of AI in cirrhotic patients, the most common cut-off used is of 550 nmol/L. In contrast, LD-SST uses 1 μg of synacthen given intravenously, and serum cortisol measured after 20 and 30 min. In the LD-SST, plasma cortisol is measured at 0, 20, and 30 min after stimulation with 1 μg corticotrophin iv. If peak cortisol exceeds 500 nmol/L, adrenal function is normal. This test seems to be more sensitive than SD-SST and evaluates better the stable cirrhotic patients (Fede et al. 2012). Most of the data in the literature have used the SD-SST, and the available data on the LD-SST are limited and not sufficient to make sound recommendation.

There are significant discrepancies between studies on the prevalence of AI in patients with liver cirrhosis, mainly because of the different tests used for diagnosis of adrenal dysfunction and the criteria applied to define AI. Thus, the prevalence of AI varies between critically ill cirrhotic patients (Table 1) and those with stable cirrhosis (Table 2). The reported prevalence of AI in critically ill cirrhotics varied between 10% and 87%. Almost all studies used SD-SST for the diagnosis of adrenal dysfunction. The prevalence of AI is strictly related to disease severity in term of Child Pugh and MELD scores. Most of these studies support the role of low HDL levels to predict the presence of AI in critically ill cirrhotic patients (Marik 2006; Etogo-Asse et al. 2012). AI is also common in patients with stable cirrhosis. However, as in critically ill cirrhotic patients, AI prevalence rate varies significantly (7–83%) depending on the diagnostic test used. In agreement with critically ill

Table 1 Prevalence of adrenal insufficiency in critically ill patients with liver cirrhosis

Reference	No. of patients and type of liver disease	Type of test performed and definition of AI	Prevalence of AI (%)
Harry et al. 2002	ALF: 45	SD-SST: baseline cortisol <250 nmol/L, Δcortisol <250 nmol/L or peak cortisol <500 nmol/L	62
Marik et al. 2005	Fulminant hepatitis: 24 CLD: 146 Recent LT: 119 History of LT: 51	LD-SST: random cortisol level <552 nmol/L, or random cortisol level <414 nmol/L or peak cortisol <552 nmol/L in nonstressed patients	33 66 92 61
Tsai et al. 2006	Cirrhosis + sepsis: 101	SD-SST: baseline cortisol <414 nmol/L, or Δcortisol <250 nmol/L, if baseline value between 414 and 918 nmol/L	51
Fernandez et al. 2006	Cirrhosis + sepsis: 25	SD-SST: baseline cortisol <414 nmol/L, or Δcortisol <250 nmol/L if baseline value between 414 and 918 nmol/L	63
Thierry et al. 2008	Cirrhosis + sepsis: 14	SD-SST: baseline cortisol <414 nmol/L, Δcortisol <250 nmol/L	77
du Cheyron et al. 2008	Critically ill cirrhotics: 50	SD-SST: baseline cortisol <414 nmol/L, or Δcortisol <250 nmol/L if baseline cortisol between 414 and 938 nmol/L	82
Arabi et al. 2010	Cirrhosis + sepsis: 75	SD-SST: Δcortisol <250 nmol/L	76
Triantos et al. 2011	Cirrhosis + variceal bleeding: 20	SD-SST and LD-SST: peak cortisol <500 nmol/L or Δcortisol <250 nmol/L	30 (SD-SST) 60 (LD-SST)
Etogo-Asse et al. 2012	ALF: 56 ACLF: 36	SD-SST: baseline cortisol <275 nmol/L and Δcortisol <250 nmol/L	48 58
Tsai et al. 2014	Cirrhosis + variceal bleeding: 143	SD-SST: baseline cortisol <10 μg/dL or peak cortisol <9 μg/dL	30
Graupera et al. 2015	Cirrhosis + variceal bleeding: 36	SD-SST: Δcortisol <250 nmol/L	22

Table 2 Prevalence of adrenal insufficiency in stable/noncritically cirrhotic patients

Reference	No. of patients and type of liver disease	Type of test performed and definition of AI	Prevalence of AI (%)
McDonald et al. 1993	Nonalcoholic liver disease: 38	IIT: reduction in maximal increments of plasma cortisol SD-SST: reduction in maximal increments of plasma cortisol	64 31
Zietz et al. 2003	Alcoholic liver disease: 36 Viral liver disease: 16	CRH: Rise of plasma ACTH < twice the baseline Peak cortisol value <550 nmol/L or an increase <250 nmol/L	42 58
Toniutto et al. 2008	Post-transplant end-stage liver disease: 87	SD-SST: peak cortisol <20 μg/dL	26
Vincent et al. 2009	Chronic liver disease: 15 Acute liver disease: 11	SD-SST: total cortisol <550 nmol/L FCI <12	46 13
Galbois et al. 2010	Alcoholic liver disease: 63 Viral liver disease: 63 Alcohol + viral: 8 Other: 4	SD-SST: baseline cortisol <250 nmol/L, and/or peak total cortisol <494 nmol/L and/or Δcortisol <250 nmol/L SD-SST: basal salivary cortisol <1.8 ng/mL and/or post- stimulation values <12.7 ng/mL and/or increase in value <3 ng/mL	33 9
Tan et al. 2010	Alcoholic liver disease: 10 Viral liver disease: 11 Alcohol + viral: 8 PBC: 3 PSC: 4 NASH: 4 Other: 3	SD-SST: peak cortisol <500 nmol/L Δcortisol <250 nmol/L Peak plasma free cortisol <33 nmol/L	39 47 12
Fede et al. 2011	Alcoholic liver disease: 29 Viral liver disease: 47 Other: 29	LD-SST: peak serum cortisol <494 nmol/L Δcortisol <250 nmol/L	38 60
Thevenot et al. 2011	Nonseptic cirrhotics: 125	SD-SST: peak cortisol <510 nmol/L	7
Triantos et al. 2011	Cirrhosis: 60	SD-SST: peak cortisol <500 nmol/L LD-SST: peak cortisol <500 nmol/L	30 48
Acevedo et al. 2013	Decompensated cirrhosis: 143	SD-SST: peak cortisol <9 μg/dL and basal cortisol <35 μg/dL	26
Jang et al. 2014	Cirrhosis: 54	SD-SST: peak cortisol <9 μg/dL and basal cortisol <35 μg/dL	24

(*continued*)

Table 2 (continued)

Reference	No. of patients and type of liver disease	Type of test performed and definition of AI	Prevalence of AI (%)
Fede et al. 2014	Stable cirrhosis: 79	LD-SST: peak cortisol <494 nmol/L FCI <12 stimulated free cortisol <33 nmol/L	34 30 29
Risso et al. 2015	Cirrhosis with ascites without sepsis or shock	SD-SST: Δcortisol <250 nmol/L or peak cortisol <500 nmol/L	39
Fede et al. 2015	Stable cirrhosis: 121	LD-SST: peak cortisol <494 nmol/L or stimulated free cortisol <33 nmol/L	38

patients, the severity of liver disease and cholesterol are predictive factors of AI (Spadaro et al. 2015).

Serum Free Cortisol

Over 90% of circulating cortisol in serum is bound to proteins, namely, corticosteroid-binding globulin (CBG) and albumin. Normally, 70% of circulating cortisol is bound to CBG, 20% is bound to albumin, and 10% exists as free cortisol. As cortisol concentration exceeds ~500 nmol/L, CBG saturates so that the biologically active free cortisol increases. At these levels, the clearance of total cortisol increases and the disappearance rate is negatively correlated with CBG. CBG has a diurnal rhythm in rats, and the metabolic clearance rate of cortisol in humans is significantly higher at 0500–1100 h compared to that at 2000–0200 h.

Reduced serum concentrations of albumin and CBG may be associated with a reduction in the bound- cortisol fraction. Therefore, serum total cortisol concentrations may be lower without altering the free biologically active hormone (Hamrahian et al. 2004; Arafah 2006).

CBG was found to be significantly decreased in cirrhotic patients compared to healthy controls. In this setting, in case of hypoalbuminemia or reduced CBG, the total cortisol is reduced while free cortisol, responsible for glucocorticoid activity on peripheral organs remains unchanged. Total cortisol, as a marker of adrenal function, may lead to overestimation of AI in patients with cirrhosis. The optimal method could be the direct evaluation of free cortisol, but its measurement is difficult in daily clinical practice. Serum free cortisol dosage is costly and time-consuming and results are usually delayed and unsuitable for guiding acute clinical decisions. Indeed, no study has yet explored the best serum free cortisol threshold for identifying patients with or without adrenal dysfunction. Up to now, the most commonly thresholds used are <50 nmol/L at baseline and <86 nmol/L after SD-SST (Hamrahian et al. 2004).

The determination of serum free cortisol (SFC) provide not only the best estimation of adrenal function but has also a prognostic value.

In hemodynamically stable cirrhotic patients, high levels of SFC are associated with a poor prognosis. Indeed the concentrations of SFC were positively correlated

with C-reactive protein (CRP) levels, suggesting that SFC increases in the context of systemic inflammation (Thevenot et al. 2012). Interestingly, the serum free cortisol tends to be higher in more severe liver disease compared to Child-Pugh A, reflecting an adrenal response to a more severe illness (Thevenot et al. 2011).

Salivary Cortisol and Surrogate Markers of Free Cortisol

Indirectly free cortisol can be calculated by Coolens equation based on total cortisol and CBG. The free cortisol index (FCI) ratio between total cortisol and CBG concentration was used as the surrogate marker for free cortisol and <12 used as cut-off (Coolens et al. 1987).

Determining salivary cortisol concentrations is an easier, noninvasive, and more reliable alternative approach for measuring serum-free cortisol concentrations, as cortisol concentrations in saliva are in equilibrium and correlate well with free cortisol concentrations. Salivary cortisol has been used as a surrogate marker of free cortisol but present limitations in cirrhosis including the high incidence of oral candidiasis, gums bleeding, and parotitis especially in alcoholics. Furthermore, the salivary gland has abundant 11β-hydroxysteroid dehydrogenase type2 activity which converts cortisol into cortisone.

In a previous study, the authors aimed to assess the prevalence of adrenal insufficiency using salivary and serum assays and to investigate the correlation between salivary, serum total and free cortisol. The authors concluded that salivary cortisol correlates strictly with free cortisol and thus better reflects adrenal function in cirrhotic patients (Galbois et al. 2010). Similarly a further study compared salivary cortisol concentrations with serum total cortisol in a large group of cirrhotic patients. Salivary cortisol concentrations were closely correlated with serum-free cortisol concentrations suggesting the potential use of salivary cortisol as a surrogate marker of free cortisol (Thevenot et al. 2011). Although the results are promising, salivary cortisol assay need to be standardize as there is no available standardized assay to measure salivary cortisol, but only “home-made” assays. It is mandatory for each laboratory to establish its own reference value.

Cortisol as Prognostic Marker of Liver Cirrhosis: Severity and Mortality

Several prognostic scores have been developed to estimate the survival of patients with liver cirrhosis. However, all the currently available prognostic scores exhibit many limitations leading to an increasing interest to find new biomarkers that may provide additional information. The prognostic assessment of cirrhotic patients can also be ascertained from cortisol levels (Di Martino et al. 2015). Adequate levels of cortisol are necessary to overcome critical illness by increasing cardiac output and vascular tonus and decreasing the release of tissue-damaging proinflammatory cytokines. However, stress-induced activation of the HPA axis may not be sufficient in patients with relative adrenal insufficiency (RAI). RAI is a condition characterized

by inappropriate plasma cortisol production by the adrenal glands relative to peripheral requirements. Several studies published over the past decade have shown that RAI is highly prevalent in cirrhotic patients with critical illnesses such as sepsis, shock and acute variceal bleeding, and in patients with decompensated cirrhosis. Furthermore, it has been shown that RAI is correlated with poor liver function and increased short-term mortality. In patients with cirrhosis and sepsis, the prevalence of adrenal insufficiency is very high; baseline cortisol and the response to corticotropin are below normal reference ranges in nearly 30% and 70% of patients, respectively.

Mean arterial pressure, serum bilirubin, vasopressor dependency, and bacteremia are independent factors prediction adrenal insufficiency in critically ill patients with cirrhosis and severe sepsis. In this context, RAI is significantly associated with septic shock, disease severity, renal failure, and hospital mortality (Fernàndez et al. 2006). The diagnosis of adrenal insufficiency in sepsis based on clinical data is very difficult. In this setting, cortisol is essential to make the diagnosis, but it is not merely a marker of adrenal function but also a prognostic tool. In fact, as is consistent with previous observations in patients with septic shock, the cortisol response is also an independent prognostic factor in patients with cirrhosis and severe sepsis (Tsai et al. 2006).

Variceal hemorrhage is a serious complication in patients with cirrhosis. In cirrhotic patients with acute gastroesophageal variceal bleeding (GEVB), a high prevalence of RAI was reported. Adrenal insufficiency is an independent predictive factor of treatment failure. In acute GEVB, the reduction of portal pressure is one of the keys to achieve hemostasis efficiently. Currently, the mainstay of pharmacological treatment lies in mesenteric vasoconstrictors, such as vasopressin and its analog, which reduce portal inflow and pressure. It has already shown that dexamethasone can dose dependently improve the hemodynamic effects of glypressin in bleeding portal hypertensive rats with adrenal insufficiency by inhibiting overproduction of cytokines and nitric oxide. Indeed, RAI is also associated with 6-week mortality through its pathophysiological links with shock, bacterial infection, and severity of liver disease (Tsai et al. 2014).

More recent data confirm the high prevalence of RAI in cirrhotic patients during severe gastrointestinal (GI) bleeding (Triantos et al. 2011). In agreement with previous data, patients with RAI have a higher risk of therapeutic failure and lower probability of survival without failure than patients with normal adrenal function (NAF). Furthermore, patients with RAI have higher infections rate at admission and during hospitalization. Previous other studies have shown that among critically and noncritically ill cirrhotic patients, those with RAI have higher probability of sepsis, renal failure, transfusion requirements, and death. Furthermore, patients with high baseline cortisol levels are more prone to develop RAI, suggesting that an adrenal exhaustion can occur in this setting (Graupera et al. 2015). Such a worse outcome of cirrhotic patients with RAI may be related to several factors. RAI is associated with a severe impairment in circulatory function that may contribute to therapeutic failure in patients with severe GI

bleeding. The presence of RAI during the course of severe bleeding may increase the risk of dislodgement of early clot by impairing the compensatory splanchnic vasoconstriction associated with hypovolemia. All these data support that in patients with cirrhosis and variceal hemorrhage there are several alterations in cortisol homeostasis. Different mechanisms explain the above alterations such as the release of inflammatory cytokines that impairs HPA axis, the adrenal hypoperfusion secondary to circulatory dysfunction, and the decreased production of cholesterol from the liver.

Also in noncritically cirrhotic patients, a significant correlation between RAI and short- and long-term mortality was shown. A prevalence of RAI of 26% was found in noncritically ill patients with cirrhosis admitted to the hospital for the treatment of acute decompensation. A relevant finding of this study was the association between RAI and the risk of new infections, severe sepsis, and type 1 hepatorenal syndrome (HRS). Indeed, the probability of death was higher in RAI patients compared with NAF. The main cause of death in these patients was acute or chronic liver failure. The second one was septic shock. In the analysis of independent risk factors for the development of death, delta cortisol together with MELD were found to be independent predictors of mortality, further supporting the prognostic role of cortisol in liver cirrhosis (Acevedo et al. 2013). In another recent study, RAI was found in 24% of noncritically cirrhotic patients. Of note, the prevalence of RAI increased with increasing severity of liver disease, with higher levels of bilirubin, a prolonged prothrombin time, and lower serum albumin and HDL cholesterol. Confirming previous data, lower HDL cholesterol levels in particular were associated with the presence of RAI. Another important finding of this study is the correlation between RAI and long-term mortality (mean follow-up 20 months) of the cirrhotic patients as an independent prognostic factor (Jang et al. 2014).

Cortisol as Marker of Systemic Inflammation

As mentioned above, highly stimulated free cortisol levels are related to poor outcome. The higher stimulated free cortisol concentrations observed in non-survivors reflect the high degree of liver insufficiency typically associated with high concentrations of proinflammatory cytokines (IL-1, IL-6 and TNF-α) and oxidative damage affecting HPA axis. High CRP levels were associated with systemic inflammation and predicted short-term mortality in severe cirrhotic patients. The involvement of IL-6 in the activation of a proinflammatory response during cirrhosis is well established and may be encouraged by sustained stimulation caused by lypopolisaccaride binding protein. The concentrations of SFC both basal and after stimulation were positively correlated with CRP concentrations. Since CRP reflects IL-6 synthesis, it is assumed that cortisol reflect systemic inflammation creating an “adrenal stress state” resulting in higher cortisol production.

Cortisol as Marker of Circulatory Dysfunction

Corticosteroids are essential for the maintenance of vascular tone; they can restore vascular responses to endogenous and exogenous vasoconstrictors, such as catecholamine, angiotensin II, endothelin, and vasopressin. Patients with adrenal insufficiency share similar hemodynamic features with patients with cirrhosis, namely, increased cardiac output, decreased peripheral vascular resistance, decreased mean arterial pressure, and hyporesponsiveness to vasopressors. Patients with cirrhosis are characterized by hyperdynamic circulation, which is closely related to the complications of liver cirrhosis. Adrenal dysfunction may contribute to cardiovascular derangement and may represent a marker of circulatory dysfunction. This phenomenon may be of clinical relevance because it represents a risk and causal factor that can be readily identified and potentially modified by steroid supplement. Furthermore, the existence of an "adrenal insufficiency- associated cardiomyopathy" has recently been hypothesized, suggesting that low glucocorticoid level causes a reduced number and downregulation of beta-adrenergic receptors and cardiotoxic fibrosis of the heart, resulting in impaired cardiac contractility and hence worsening of the circulatory and renal dysfunction (Theocharidou et al. 2012). In cirrhotic patients, AI is associated with a significantly lower mean arterial pressure and higher plasma renin activity and norepinephrine concentration (Acevedo et al. 2013). Adrenal failure attenuates the vascular effect of angiotensin-II, norepinephrine, and vasopressin in decompensated cirrhosis, further activating sympathetic tone. Since sympathetic hyperactivity impairs intestinal motility and immunity, a vicious circle favoring bacterial overgrowth, bacterial translocation, bacterial infections, and/or systemic inflammatory response can ensue. RAI can also be involved in the development of an excessive compensatory anti-inflammatory response. In long-standing decompensated cirrhosis, adrenal glands respond poorly to hypotensive stress, also because of the inhibitory effect of cytokines on CRH and ACTH and adrenal inflammation, preventing a rise in cortisolemia and reducing the vascular effects of vasoconstrictors.

Cortisol as Marker of Bacterial Translocation and Occult Infections

Adrenal activation with increased hormonal output is associated with an increased risk of mortality in noncritically ill patients with advanced cirrhosis. An explanation of this adrenal activation may be an underlying chronic inflammatory state, caused by microbial translocation from the gut lumen to the organism promoted by portal hypertension. The presence of microbial DNA correlated with adrenal function and mortality (Risso et al. 2015). In critically cirrhotic patients, bacteremia was an independent factor predicting impaired adrenal function. The presence of viable bacteria in the blood may reflect a higher bacterial load in a more immunocompromised host. The baseline and peak cortisol levels as well as the cortisol increment

were all significantly lower in the bacteremic group, implying altered adrenal synthesis and responsiveness in this specific subset of patients (Tsai et al. 2006). It is well known that circulatory dysfunction and the secondary activation of the sympathetic nervous system impairs several defensive mechanisms against enteric infections. Overactivation of the sympathetic nervous system inhibits intestinal motility and increases bacterial overgrowth. The increased release of catecholamines from adrenergic terminals exerts potent immunosuppressive actions including inhibition of chemotaxis/migration and phagocytosis of bacteria by neutrophils and monocytes. Finally, catecholamines are released into the intestinal lumen, where they interact with specific bacterial receptors and increase bacterial growth, adherence to the mucosa, penetration into the interstitial space and lymphatics within the intestinal wall, and virulence. The net effect of all these alterations is an increased translocation of bacteria and bacterial products from the intestinal lumen to the submucosal lymphatics and then to the systemic circulation, giving rise to spontaneous bacterial infections and systemic inflammatory response. Since adrenal dysfunction is associated with reduction in the vascular effect of the renin-angiotensin and the sympathetic nervous systems, the compensatory increase in the adrenergic tone could contribute to increase the risk of bacterial infection.

24H. Cortisol Rhythm Change as Marker of Hepatic Encephalopathy

CRH modulates sleep. Exogenous CRH increases electroencephalograms (EEG) frequency in rats, decreases slow wave sleep (SWS), increases light sleep and awakenings in humans, and may decrease rapid eye movement (REM) sleep. Cortisol levels in serum are reduced in cirrhotic patients at all times measured correlating with the severity of liver disease and of the alterations of the EEG. Circadian rhythm of cortisol level in plasma is also altered in cirrhotic patients with delayed onset and shortened peak duration, suggesting that alterations in glucocorticoid hormones could be involved in altered circadian rhythms in patients with liver disease and hepatic encephalopathy (HE).

A considerable delay in the onset of the cortisol rhythm and abnormalities in the duration of the peak were observed in cirrhotic patients, in parallel with the degree of hepatic dysfunction. While the exact pathophysiology of cortisol timing alterations in patients with cirrhosis is unknown, they are most likely of cerebral origin. Cortisol, like melatonin, is a direct marker of the phase of the hypothalamic clock, which regulates the diurnal rhythm of circadian hormones in relation to light and dark cues. It has previously been shown that patients with cirrhosis exhibit abnormalities in the sensitivity of the hypothalamic clock to light, which may explain the abnormalities in both melatonin and cortisol rhythms. Hyperammonemia is a main factor in the induction of the neurological disturbances in HE. Sleep is also exquisitely sensitive to ammonia levels in cirrhotic patients. Hyperammonemia induced in cirrhotic patients is associated with changes in the EEG, increased sleepiness, and more superficial sleep. The circadian rhythms of motor activity and plasma levels of corticosterone are altered in rats with chronic hyperammonemia similar to that

present in patients with liver cirrhosis. Chronic hyperammonemia impairs adrenal function and ACTH-induced corticosterone release, leading to reduced feedback modulation of the HPA axis. Reduced release of corticosterone from adrenal glands contribute to the impairment of circadian rhythms of motor activity in hyperammonemia and HE. It was shown that impairment of circadian rhythms and motor activity may be corrected in hyperammonemia by pharmacological treatment using a single daily injection of corticosterone (Montagnese et al. 2011; Montagnese et al. 2010).

Potential Applications to Prognosis, Other Diseases or Conditions

Cortisol plays a crucial role in many processes relevant to metabolic disturbances such as hypertension, hyperlipidemia, and central obesity, which predispose individuals to prediabetes and overt type-2 diabetes (T2D). In cross-sectional studies with healthy subjects, raised cortisol concentrations assessed from plasma samples and 24-h urinary free samples have been associated with raised plasma glucose and insulin resistance. Recent evidences established an association between cortisol and new-onset T2D; cortisol levels could be a potential biomarker of future glucose disturbance (Hackett et al. 2016).

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the global obesity and metabolic disease epidemic. The exact pathophysiology remains unclear with a multi-hit model that has been proposed including propensity to lipid accumulation, triggers to inflammation and fibrosis and impaired liver regenerative capacity. Cortisol has been implicated in the pathogenesis of NAFLD, and the liver itself has been shown to produce significant amounts of cortisol into the splanchnic circulation. Prolonged and excessive exposure to glucocorticoids can have detrimental consequences including NAFLD (Woods et al. 2015). Indeed, cortisol levels could represent an attractive biomarker, not only for the diagnosis but also to assist the clinicians in therapeutic decisions and monitoring.

Summary Points

- This chapter focuses on cortisol, an hormone produced by adrenal glands under control of the hypothalamus-pituitary-adrenal axis.
- Recently, cortisol was proposed as a potential biomarker of liver disease in terms of severity and prognosis.
- The data show that adrenal insufficiency is a common complication of liver cirrhosis both in critically and noncritically ill patients. Adrenal function is strictly correlated with liver disease severity, complications such as infections, sepsis, hemodynamic impairment, and mortality.
- Up to now, the diagnosis is based on laboratory tests because the clinical characteristics of cirrhotic patients with hepato-adrenal syndrome are unknown and difficult to define. In critically ill cirrhotic patients, hypotension refractory to

vasopressors and fluid resuscitation is the most important clinical sign. In contrast, in stable cirrhotic patients, the diagnosis of AI based on clinical grounds is almost impossible because of the lack of typical signs and symptoms.

- Although the diagnosis of adrenal insufficiency in liver cirrhosis is clinically relevant, there is no consensus about the most accurate diagnostic method to recommend in this setting.

References

Acevedo J, Fernàndez J, Prado V, et al. Relative adrenal insufficiency in decompensated cirrhosis: relationship to short-term risk of severe sepsis, hepatorenal syndrome, and death. Hepatology. 2013;58(5):1757–65.

Arabi YM, Aljumah A, Dabbagh O, et al. Low-dose hydrocortisone in patients with cirrhosis and septic shock: a randomized controlled trial. CMAJ. 2010;182(18):1971–7.

Arafah BM. Hypothalamic pituitary adrenal function during critical illness: limitations of current assessment methods. J Clin Endocrinol Metab. 2006;91(10):3725–45.

Biddie SC, Conway-Campbell BL, Lightman SL. Dynamic regulation of glucocorticoid signaling in health and disease. Rheumatology (Oxford). 2012;5(3):403–12.

Bornstein SR. Predisposing factors for adrenal insufficiency. N Engl J Med. 2009;360:2328–39.

Cicognani C, Malavolti M, Morselli-Labate AM, et al. Serum lipid and lipoprotein patterns in patients with liver cirrhosis and chronic active hepatitis. Arch Intern Med. 1997;157(7):792–6.

du Cheyron D, Bouchet B, Cauquelin B, et al. Hyperreninemic hypoaldosteronism syndrome, plasma concentrations of interleukin-6 and outcome in critically ill patients with liver cirrhosis. Intensive Care Med. 2008;34(1):116–24. Epub 2007 Sep 29.

Coolens JL, Van Baelen H, Heyns W. Clinical use of unbound plasma cortisol as calculated from total cortisol and corticosteroid-binding globulin. J Steroid Biochem. 1987;26:197–292.

Di Martino V, Weil D, Cervoni JP, et al. New prognostic markers in liver cirrhosis. World J Hepatol. 2015;7(9):1244–50.

Etogo-Asse FE, Vincent RP, Hughes SA, et al. High density lipoprotein in patients with liver failure; relation with sepsis, adrenal function and outcome of illness. Liver Int. 2012;32:128–36.

Fede G, Spadaro L, Privitera G, et al. Hypothalamus-pituitary dysfunction is common in patients with stable cirrhosis and abnormal low dose synacthen test. Dig Liver Dis. 2015;47(12):1047–51. doi:10.1016/j.dld.2015.08.006. Epub 2015 Aug 19.

Fede G, Spadaro L, Tomaselli T, et al. Adrenocortical dysfunction in liver disease: a systematic review. Hepatology. 2012;55:1282–91.

Fede G, Spadaro L, Tomaselli T, et al. Comparison of total cortisol, free cortisol, and surrogate markers of free cortisol in diagnosis of adrenal insufficiency in patients with stable cirrhosis. Clin Gastroenterol Hepatol. 2014;12(3):504–12.

Fede G, Spadaro L, Tomaselli T, Privitera G, Piro S, Rabuazzo AM, et al. Assessment of adrenocortical reserve in stable patients with cirrhosis. J Hepatol 2011;54(2):243–50.

Fernàndez J, Escorsell A, Zabalza M, et al. Adrenal insufficiency in patients with cirrhosis and septic shock: effect of treatment with hydrocortisone on survival. Hepatology. 2006;44:1288–95.

Galbois A, Rudler M, Massard J, et al. Assessment of adrenal function in cirrhotic patients: salivary cortisol should be preferred. J Hepatol. 2010;52:839–45.

Graupera I, Pavel O, Hernandez-Gea V, et al. Relative adrenal insufficiency in severe acute variceal and non-variceal bleeding: influence on outcomes. Liver Int. 2015;35:1964–73.

Hackett RA, Kivimaki M, Kumari M, et al. Diurnal cortisol patterns, future diabetes, and impaired glucose metabolism in the Whitehall II cohort study. J Clin Endocrinol Metab. 2016; 101(2):619–25.

Hamrahian AH, Oseni TS, Arafah BM. Measurements of serum free cortisol in critically ill patients. N Engl J Med. 2004;350:1629–38.
Harry R, Auzinger G, Wendon J. The clinical importance of adrenal insufficiency in acute hepatic dysfunction. Hepatology 2002;36(2):395–402.
Jang JY, Kim TY, Sohn JH, et al. Relative adrenal insufficiency in chronic liver disease: its prevalence and effects on long- term mortality. Aliment Pharmacol Ther. 2014;40:819–26.
Kadmiel M, Cidlowski JA. Glucocorticoid receptor signaling in health and disease. Trends Pharmacol Sci. 2013;34(9):518–30.
Marik PE. Adrenal-exhaustion syndrome in patients with liver disease. Intensive Care Med. 2006;32(2):275–80.
Marik PE, Gayowski T, Starzl TE et al. The hepatoadrenal syndrome: a common yet unrecognized clinical condition. Crit Care Med. 2005;33(6):1254–9.
McDonald JA, Handelsman DJ, Dilworth P, et al. Hypothalamic-pituitary adrenal function in end-stage non-alcoholic liver disease. J Gastroenterol Hepatol. 1993;8(3):247–53.
Montagnese S, Middleton B, Mani AR, et al. On the origin and the consequences of circadian abnormalities in patients with cirrhosis. Am J Gastroenterol. 2010;105(8):1773–81.
Montagnese S, Middleton B, Mani AR, et al. Changes in the 24-h plasma cortisol rhythm in patients with cirrhosis. J Hepatol. 2011;54(3):588–90.
Oakley RH, Cidlowski JA. The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. J Allergy Clin Immunol. 2013;132(5):1033–44.
Ramamoorthy S, Cidlowski JA. Exploring the molecular mechanisms of glucocorticoid receptor action from sensitivity to resistance. Endocr Dev. 2013;24:41–56.
Risso A, Alessandria C, Mezzabotta L, et al. Adrenal function and microbial DNA in noninfected cirrhotic patients with ascites: relationship and effect on survival. Dig Liver Dis. 2015; 47(8):702–8.
Spadaro L, Noto D, Privitera G, Tomaselli T, Fede G, Scicali R, Piro S, Fayer F, Altieri I, Averna M, Purrello F. Apolipoprotein AI and HDL are reduced in stable cirrhotic patients with adrenal insufficiency: a possible role in glucocorticoid deficiency. Scand J Gastroenterol. 2015;50 (3):347–54.
Tan T, Chang L, Woodward A, McWhinney B, Galligan J, Macdonald GA, et al. Characterising adrenal function using directly measured plasma free cortisol in stable severe liver disease. J Hepatol 2010;53(5):841–8.
Theocharidou E, Krag A, Bendtsen F, et al. Cardiac dysfunction in cirrhosis- does adrenal function play a role? A hypothesis. Liver Int. 2012;32(9):1327–32.
Thevenot T, Borot S, Remy-Martin A, et al. Assessment of adrenal function in cirrhotic patients using concentration of serum-free and salivary cortisol. Liver Int. 2011;31:425–33.
Thevenot T, Dorin R, Monnet E, et al. High serum levels of free cortisol indicate severity of cirrhosis in hemodynamically stable patients. J Gastroenterol Hepatol. 2012;27:1596–601.
Thierry S, Giroux Leprieur E, Lecuyer L, et al. Echocardiographic features, mortality, and adrenal function in patients with cirrhosis and septic shock. Acta Anaesthesiol Scand. 2008;52(1): 45–51. Epub 2007 Nov 8.
Toniutto P, Fabris C, Fumolo E, et al. Prevalence and risk factors for delayed adrenal insufficiency after liver transplantation. Liver Transpl. 2008;14:1014–9.
Triantos CK, Marzigie M, Fede G, et al. Critical illness-related corticosteroid insufficiency in patients with cirrhosis and variceal bleeding. Clin Gastroenterol Hepatol. 2011;9(7):595–601.
Trifan A, Chiriac S, Stanciu C. Update on adrenal insufficiency in patients with liver cirrhosis. World J Gastroenterol. 2013;19(4):445–56.
Tsai MH, Peng YS, Chen YC, et al. Adrenal insufficiency in patients with cirrhosis, severe sepsis and septic shock. Hepatology. 2006;43:673–81.
Tsai MH, Huang HC, Peng YS, et al. Critical illness-related corticosteroid insufficiency in cirrhotic patients with acute gastroesophageal variceal bleeding: risk factors and association with outcome. Crit Care Med. 2014;42(12):2546–55.

Vincent RP, Etogo-Asse FE, Dew T, et al. Serum total cortisol and free cortisol index give different information regarding the hypothalamus-pituitary-adrenal axis reserve in patients with liver impairment. Ann Clin Biochem. 2009;46(Pt 6):505–7.

Walker BR, Andrew R. Tissue production of cortisol by 11β-hydroxysteroid dehydrogenase type 1 and metabolic disease. Ann N Y Acad Sci. 2006;1083:165–84.

Woods CP, Hazlehurst JM, Tomlinson JW. Glucocorticoids and non-alcoholic fatty liver disease. J Steroid Biochem Mol Biol. 2015;154:94–103.

Zhou J, Cidlowski JA. The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. Steroids. 2005;70:407–17.

Zietz B, Lock G, Plach B, et al. Dysfunction of the hypothalamic-pituitary-glandular axes and relation to Child-Pugh classification in male patients with alcoholic and virus-related cirrhosis. Eur J Gastroenterol Hepatol. 2003;15(5):495–501.

Serum Sialic Acid as a Biomarker in Liver Disease

19

Ewa Gruszewska and Lech Chrostek

Contents

Abstract

Sialic acid (SA) is the nine-carbon *N*-acetylated derivatives of neuraminic acid. It is mostly found in the terminal position on nonreducing end of oligosaccharide chains of glycoproteins and glycolipids on the surface of cells and molecules. Due to this position and unique structural features, sialic acid is one of the most important molecules for life. The synthesis, catabolism, and attaching of this molecule to the oligosaccharide chains of proteins and lipids take place in the liver. Therefore, it has been hypothesized that the liver function can affect the concentration of protein-bound sialic acid (PBSA), lipid-bound sialic acid (LSA),

E. Gruszewska (✉) • L. Chrostek
Department of Biochemical Diagnostics, Medical University of Bialystok, Bialystok, Poland
e-mail: gr_ewa@interia.pl; chrostek@umb.edu.pl

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_11

and free sialic acid (FSA). The changes in liver status play an important role in the pathogenesis and progression of various liver diseases. In different liver diseases, the activity of enzymes involved in glycosylation is also changed. For the aberrant glycosylation of proteins and lipids in liver diseases, different glycosyltransferases are responsible. Therefore, the changes in the glycan structure of glycoproteins and glycolipids can be characteristic for the specific liver disease and could be potentially used in the diagnostics of liver pathology. The estimation of serum sialic acid level may be helpful as a biomarker of liver diseases. This study summarizes previously data about the changes in sialic acid concentrations in liver disease.

Keywords

Sialic acid • Glycosylation • Sialylation • Liver diseases • Biomarkers

List of Abbreviations

A2M	Alpha2-macroglobulin
AAT	Alpha1-antitrypsin
AFP	Alpha-fetoprotein
AFP-L3	Fucosylated variant of alpha-fetoprotein
AGP	Alpha1-acid glycoprotein
ALD	Alcoholic liver disease
Apo-B	Apolipoprotein B
Apo-J	Apolipoprotein J
CDT	Carbohydrate-deficient transferrin
CER	Ceruloplasmin
CMP-Neu5Ac	Cytidine monophosphate *N*-acetylneuraminic acid
CMP-NeuAc synthetase	Cytidine monophosphate *N*-acetylneuraminic acid synthetase
CRP	C-reactive protein
CTP	Cytidine triphosphate
FSA	Free sialic acid
GnT-III	Glycosyltransferase III
GnT-V	Glycosyltransferase V
HCC	Hepatocellular carcinoma
HP	Haptoglobin
IL-1	Interleukin-1
IL-6	Interleukin-6
LSA	Lipid-bound sialic acid
ManNAc	*N*-Acetyl D-mannosamine
ManNAc-6-kinase	*N*-Acetylmannosamine 6-kinase
ManNAc-6-P	*N*-Acetylmannosamine-6-phosphate
Neu5,9Ac	*N*-Acetyl-9-*O*-acetylneuraminic acid
Neu5Ac	*N*-Acetylneuraminic acid

Neu5Ac9-P	*N*-Acetylneuraminic acid-9-phosphate
Neu5Ac-9-P-phosphatase	*N*-Acetyl-D-neuraminyl-9-phosphatase
Neu5Ac-9-P-synthetase	*N*-Acetyl-D-neuraminyl-9-phosphate synthetase
Neu5Glc	*N*-Glycolylneuraminic acid
PBSA	Protein-bound sialic acid
PEP	Phosphoenolpyruvate
SA	Sialic acid
TNF-α	Tumor necrosis factor alpha
TNM	Tumor-node-metastases classification
TRF	Transferrin
TSA	Total sialic acid
UDP-GlcNAc	Uridine diphosphate *N*-acetylglucosamine
UDP-GlcNAc-2-epimerase	Uridine diphosphate *N*-acetylglucosamine 2-epimerase

Key Facts of Sialic Acid

- The term "sialic acid" comes from the Greek "*sialos*" – saliva.
- The term "sialic acid" was first introduced in the year 1952 by Gunnar Blix, a Swedish biochemist.
- First, sialic acid was discovered as a cellular receptor for influenza viruses.
- Sialic acids represent a family of sugar molecules, currently including about 50 members.
- In human sialic acid can exist as a free form or is associated with proteins and lipids.
- The serum concentration of sialic acid changes in many diseases.
- Sialic acid is also a marker of chronic alcohol consumption.

Definitions of Word and Terms

Glycolipids	Lipids with covalently attached carbohydrates.
Glycoproteins	Proteins with covalently attached carbohydrates.
Glycosylation	Kind of reaction in which a carbohydrate is attached to another molecule.
Glycosyltransferases	Proteins with an enzymatic activity that catalyze the attachment of carbohydrate to another molecule.
Liver disease	Is a general term for any damage of the liver.
Sialic acid	Nine-carbon sugar.
Sialidases	Proteins with an enzymatic activity that catalyze cleavage of sialic acid from another molecule.
Sialylation	Kind of reaction in which the sialic acid is attached to another molecule.

Introduction

Due to the fact that sialic acid (SA) is an ubiquitous molecule in the organism, it plays an important role both in physiological and pathological processes. In previous literature, there are many reports indicated that change in serum sialic acid concentrations may be a potential marker for several diseases. For example, the differences in sialic acid level have been reported in inflammatory diseases, diabetes mellitus, bacterial infections, rheumatoid arthritis, and malignant diseases. This review focused on sialic acid as a biomarker of liver disease. It is well known that the variations in serum sialic acid concentrations in liver disease occur, but there are differences in previously published data.

A Few Words About the Sialic Acid

Structure and Synthesis of Sialic Acid

Sialic acids represent a family of monosaccharides currently including about 50 natural members (Bork et al. 2009). They are the *N*- or *O*-acetylated derivatives of neuraminic acid (5-amino-3,5-dideoxy-2-nonulosonic acid) (Schauer and Kamerling 1997; Traving and Schauer 1998). The most common sialic acids are *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Glc), and *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac) (Table 1) (Sillanaukee et al. 1999). *N*-Acetylneuraminic acid is composed of nine carbon atoms and the carboxyl group located at the C2 anomeric carbon atom (Fig. 1) (Traving and Schauer 1998; Varki 2008). The carboxyl acid group gives a negative charge of sialic acid.

The Neu5Ac is synthesized in the cytosol in a four-step process with the participation of four enzymes (Fig. 2) (Schauer and Kamerling 1997; Bork et al. 2009). In the first step, the UDP-*N*-acetylglucosamine (UDP-GlcNAc) is converted to *N*-acetyl D-mannosamine (ManNAc) with removal of the UDP moiety and epimerization of the carbohydrate carried out by two enzymes: UDP-*N*-acetylglucosamine 2-epimerase (UDP-GlcNAc-2-epimerase) and *N*-acetylmannosamine 6-kinase (ManNAc-6-kinase). In the second step, the same enzymes phosphorylate the ManNAc to *N*-acetylmannosamine-6-phosphate (ManNAc-6-P). The third reaction, condensation of ManNAc-6-P with phosphoenolpyruvate (PEP) initiated by *N*-acetyl-D-neuraminyl-9-phosphate synthetase (Neu5Ac-9-P-synthetase), and the product of this reaction is *N*-acetylneuraminic acid-9-phosphate (Neu5Ac9-P). In the last reaction, this compound is dephosphorylated by *N*-acetyl-D-neuraminyl-9-phosphatase (Neu5Ac-9-P-phosphatase) to produce the *N*-acetylneuraminic acid (Neu5Ac). Then, Neu5Ac is modified to the other members of the sialic acid family or is activated to CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) in the nucleus (Bork et al. 2009). The activation of sialic acid occurs by attaching of Neu5Ac to cytidine triphosphate (CTP). This reaction is catalyzed by CMP-*N*-acetylneuraminic

Table 1 The most common derivatives of neuraminic acid. *R4, R5, R7, R8, R9* radicals at carbons 4, 5, 7, 8, 9, *A* acetyl, *G* glycolyl, *L-L* L-lactyl, *Ph* phosphate, *M* methyl, *S* sulfate, *L* lactyl, *H* hydrogen (Sillanaukee et al. 1999)

Name of sialic acid	R4	R5	R7	R8	R9
N-Acetylneuraminic acid	H	A	H	H	H
N-Acetyl-4-*O*-acetylneuraminic acid	A	A	H	H	H
N-Acetyl-7-*O*-acetylneuraminic acid	H	A	A	H	H
N-Acetyl-8-*O*-acetylneuraminic acid	H	A	H	A	H
N-Acetyl-9-*O*-acetylneuraminic acid	H	A	H	H	A
N-Acetyl-4,9-di-*O*-acetylneuraminic acid	A	A	H	H	A
N-Acetyl-7,9-di-*O*-acetylneuraminic acid	H	A	A	H	A
N-Acetyl-8,9-di-*O*-acetylneuraminic acid	H	A	H	A	A
N-Acetyl-7,8,9-tri-*O*-acetylneuraminic acid	H	A	A	A	A
N-Acetyl-9-*O*-L-lactylneuraminic acid	H	A	H	H	L-L
N-Acetyl-4-*O*-acetyl-9-*O*-lactylneuraminic acid	A	A	H	H	L
N-Acetyl-8-*O*-methylneuraminic acid	H	A	H	L	H
N-Acetyl-8-*O*-sulfoneuraminic acid	H	A	H	S	H
N-Acetyl-9-*O*-phosphoroneuraminic acid	H	A	H	H	Ph
N-Acetyl-2-deoxy-2,3-dehydroneuraminic acid	H	A	H	H	H
N-Glycolylneuraminic acid	H	G	H	H	H
N-Glycolyl-4-*O*-acetylneuraminic acid	A	G	H	H	H
N-Glycolyl-7-*O*-acetylneuraminic acid	H	G	A	H	H
N-Glycolyl-9-*O*-acetylneuraminic acid	H	G	H	H	A
N-Glycolyl-7,9-di-*O*-acetylneuraminic acid	H	G	A	H	A
N-Glycolyl-8,9-di-*O*-acetylneuraminic acid	H	G	H	A	A
N-Glycolyl-7,8,9-tri-*O*-acetylneuraminic acid	H	G	A	A	A
N-Glycolyl-8-*O*-methylneuraminic acid	H	G	H	L	H
N-Glycolyl-8-*O*-sulfoneuraminic acid	H	G	H	S	H

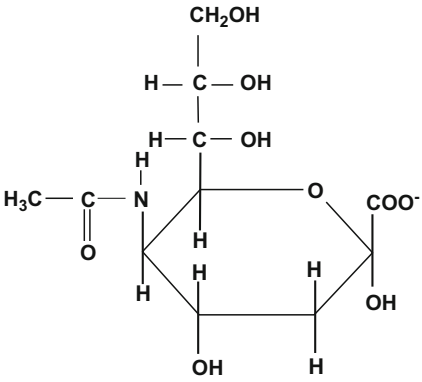

Fig. 1 Structure of *N*-acetylneuraminic acid

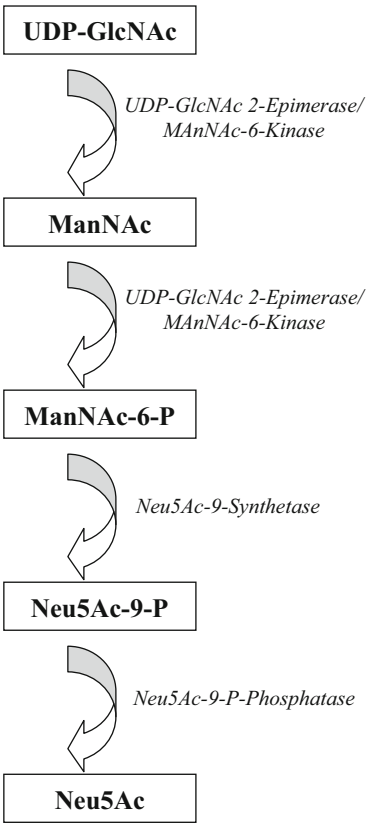

Fig. 2 The biosynthesis of sialic acid. *UDP-GlcNAc* uridine diphosphate *N*-acetylglucosamine, *ManNAc N*-acetyl D-mannosamine, *ManNAc-6-kinase N*-acetylmannosamine 6-kinase, *Neu5Ac9-P N*-acetylneuraminic acid-9-phosphate, *Neu5Ac N*-acetylneuraminic acid

acid synthetase (CMP-NeuAc synthetase) and the product is CMP-Neu5Ac. Then, the specific sialyltransferases from the Golgi apparatus attach Neu5Ac residues to oligosaccharides.

Occurrence of Sialic Acid

In the human organism, sialic acid mainly occupies the nonreducing terminal position on the carbohydrate chains of glycoproteins and glycolipids (Schauer and Kamerling 1997; Sillanaukee et al. 1999). Moreover, sialic acid may also exist as a free form, but this form is scarce in the human body (Waters et al. 1992; Sillanaukee et al. 1999). Thus, the total sialic acid (TSA) is a sum of protein-bound SA (PBSA), lipid-bound SA (LSA), and free SA (FSA). Sialic acid is widely distributed in mammal tissues, e.g., stomach, cervix, throat, colon, etc., and also in body fluids, such as blood plasma, urine, saliva, sweat, cerebrospinal fluid, breast milk, and gastric juice (Sillanaukee et al. 1999). The most common (about 80%) derivatives of sialic acid in human blood is Neu5Ac and less frequent (about 20%) is Neu5Ac9Lt (Sillanaukee et al. 1999). The

major form of sialic acid in the tissues is Neu5Ac (Sillanaukee et al. 1999; Nigam et al. 2006). Most of the serum sialic acid is bounded to glycoproteins such as fibrinogen, transferrin (TRF), haptoglobin (HP), ceruloplasmin (CER), alpha1-acid glycoprotein (AGP), and alpha1-antitrypsin (AAT) (Nigam et al. 2006). The content of sialic acid in human glycoproteins is usually between 3% and 7% (Sillanaukee et al. 1999). Moreover, sialic acid is also an important structural component of cellular membranes of red blood cells, white blood cells, and platelets (Nigam et al. 2006).

Biological Function of Sialic Acid

Sialic acid serves many important biological functions (Traving and Schauer 1998; Sillanaukee et al. 1999). First of all, it stabilizes the conformation of glycoproteins and cellular membranes, confers a negative charge for them, affects physicochemical properties and functions of glycoproteins, and determines survival of glycoproteins in blood circulation (the serum half-life of glycoproteins) (Sillanaukee et al. 1999; Varki 2008). It also affects the transmembrane transportation mechanisms. Due to the negative charge, sialic acid is involved in the binding and transport of positively charged molecules (e.g., Ca^{2+}) (Traving and Schauer 1998). Due to the final position in the oligosaccharide chains, SA is involved in the recognition and interaction between cells and molecules and also can be a chemical messenger in tissues and body fluids (Traving and Schauer 1998; Sillanaukee et al. 1999). It also serves as a component of cell surface receptors for many endogenous substances, such as hormones and cytokines, and also for many pathogens, such as viruses, bacteria, and toxins (Schauer and Kamerling 1997; Traving and Schauer 1998). Sialic acid affects the adhesion and immunogenicity of the cells too. It is also important for masking antigenic determinants or epitopes. It is well known that the receptors of the immune system, e.g., T- and B-cell receptors, often prefer nonsialylated structures (Bork et al. 2009). Sialic acid also masks the cells and molecules. An example may be a red blood cell. The lifetime of erythrocytes is around 120 days. Sialic acid is removed from erythrocyte surface by the sialidases and reveals the galactose residue. The unmasked erythrocytes are bound to macrophages and phagocytosed. In this way malignant cells may be eliminated; therefore the oversialylation of tumor cells protects these cells from humoral and cellular defense response. Thus, sialic acid plays a dual role; it is necessary for the protection of life but is also utilized during pathological states, e.g., by infectious microorganisms. So, the maintenance of the normal amount of sialic acid is necessary to the physiological functions of organisms.

Diagnostic Role of Sialic Acid

The serum concentration of sialic acid may increase in many diseases including cancer, inflammatory diseases (e.g., pneumonia, bacterial infections, rheumatoid arthritis), diabetes, chronic liver diseases, and chronic alcohol abuse (Stefenelli et al. 1985; Sillanaukee et al. 1999). Thus, in clinical pathology, the measurements

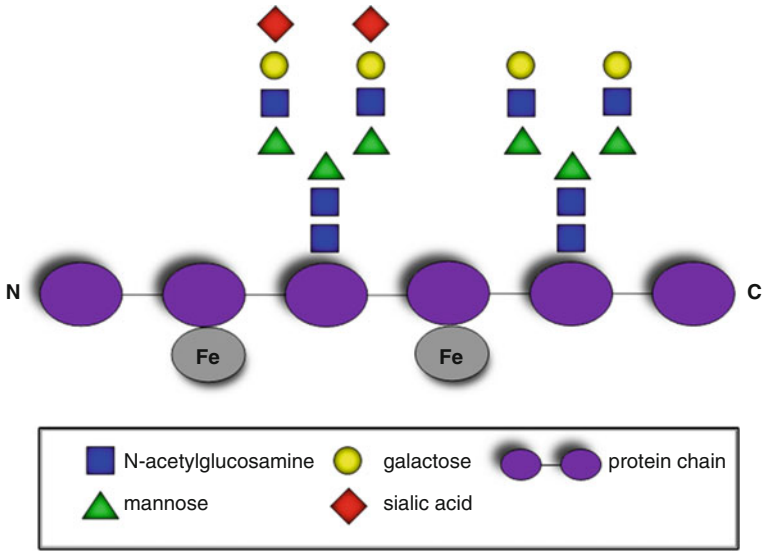

Fig. 3 Structure of carbohydrate-deficient transferrin

of sialic acid in the body fluids are used to predict the risk of various diseases. Some sialylated molecules may be detected in the blood serum as a marker of cancer progression (Sillanaukee et al. 1999; Varki 2008). The reason for this is an increased level of serum sialic acid in many types of cancers (e.g., ovarian, brain, lung, cervical, stomach, colorectal, etc.). There are many reports suggesting the association of the changes in sialylation with malignant transformation. Some data indicate that sialic acid concentrations could be elevated in cancer patients before clinical symptoms. The changed sialic acid structures and linkages are associated with progression and poor prognosis of tumors and correlated with the degree of metastasis. There are also many reports which have shown the normalization of sialic acid levels after treatment of cancer. Due to the fact that sialic acid is a component of acute-phase proteins, its concentration may also reflect an acute-phase reaction (Sillanaukee et al. 1999). On the other hand, desialylation of some structures can be also used as a marker of different diseases. An example may be a carbohydrate-deficient transferrin (CDT), which is used as a screening marker both for chronic alcohol abuse and for congenital disorders of glycosylation (Fig. 3) (Sillanaukee et al. 1999; Varki 2008). Sialic acid is also a risk factor of cardiovascular disease in diabetic patients.

Alteration in Glycosylation/Sialylation in Different Liver Diseases

The biochemical alteration in serum sialic acid levels has been assumed as a potential marker for several diseases. Previous reports have indicated that changes in the sialic acid concentrations were related, e.g., with inflammatory diseases,

diabetes mellitus, bacterial infections, rheumatoid arthritis, and malignant diseases (Nigam et al. 2006; Varki 2008; Chrostek et al. 2014). The interest in using sialic acid as a marker of acute and chronic liver disease is associated with the fact that synthesis, catabolism, and attaching to the carbohydrate chains of glycoproteins and glycolipids take place in the liver (Li and Chen 2012). Therefore, liver function influences the serum sialic acid concentrations. The variations in serum total sialic acid concentrations in liver disease have been described, but there are differences in previously published data (Kongtawelert et al. 2003; Arif et al. 2005; Cylwik et al. 2010; Chrostek et al. 2011; Gruszewska et al. 2014). This is the result of the fact that the glycosylation (especially sialylation) patterns of glycoproteins and glycolipids are highly variable and depend on the liver status.

Alterations in Glycosylation in Liver Diseases

Alteration in glycosylation during alcoholic liver disease (ALD) concerns many glycoproteins, such as transferrin, haptoglobin, ceruloplasmin, alpha2-macroglobulin (A2M), alpha1-acid glycoprotein, and alpha1-antitrypsin (Lakshman et al. 1999; Tsutsumi and Takase 2001; Blomme et al. 2009). The consequence of alcohol abuse is the inhibition of protein glycosylation in the liver by decrease in glycosyltransferase activity (enzymes which bind the oligosaccharide chains to the glycoprotein), such as galactosyltransferase, *N*-acetylglucosaminyltransferase, mannosyltransferase, and sialyltransferase, and increase in plasma and membrane sialidases (enzymes which cut terminal sialic acid residues from glycoproteins) (Blomme et al. 2009; Chrostek and Cylwik 2011). As a result of impaired synthesis of glycoproteins, increased serum levels of sialic acid and concentrations of glycoproteins with a reduced amount of SA or completely devoid of it, such as carbohydrate-deficient transferrin, were observed. Desialylation is the most important change observed in alcoholic liver disease. Because chronic alcohol consumption alters the normal microheterogeneity pattern of transferrin, the CDT is the most commonly used marker of chronic alcohol abuse (Chrostek et al. 2006). The second glycoprotein which could become a marker of ALD, especially alcoholic cirrhosis, has become haptoglobin (Blomme et al. 2009; Chrostek and Cylwik 2011). The changes in haptoglobin glycosylation depend on increased fucosylation and increased branching of N-glycans. The alterations in branching were determined by an increased *N*-acetylglucosamine content in the haptoglobin molecule. This change is not specific for alcoholic cirrhosis and can be observed also in chronic alcohol consumption without cirrhosis and in primary biliary cirrhosis. In turn, the hyperfucosylation of haptoglobin is a consequence of increased amount of fucose residues attached to the subterminal *N*-acetylglucosamine by alpha1,3-branching. This reaction is catalyzed by alpha1,3-fucosyltransferase, whose increased activity is correlated with elevated serum concentration of haptoglobin. The chronic alcohol consumption inhibits also the sialylation of apolipoprotein J (apo-J) (Gosh et al. 2001; Chrostek and Cylwik 2011). The degree of apolipoprotein J sialylation is measured by apolipoprotein J sialylation index (ratio of sialic acid concentration to

apolipoprotein J concentration – SA/apo-J), which is a seven times more sensitive marker of alcohol abuse than the transferrin sialylation (Gosh et al. 2001). It has been shown that the values of apolipoprotein J sialylation index depend on a daily dose of alcohol consumption, period of abstinence, and detoxification.

The most common alteration in bile-related liver diseases is fucosylation of N-glycans of some glycoproteins, such as haptoglobin, alpha1-antitrypsin, and alpha1-acid glycoprotein (Nakagawa et al. 2006; Chrostek and Cylwik 2011). These glycoproteins were more fucosylated in the bile than that in serum. Possibly, this could be a signal for secretion of these glycoproteins into bile ducts in the liver. The major reason of this disorder may be the increased expression of alpha1,6-fucosyltransferase.

In fatty liver disease, the aberrant glycosylation of apolipoprotein B (apo-B) is observed (Blomme et al. 2009). The changes in apo-B glycosylation disturb its function. The result of this is decreased release of lipoproteins containing apolipoprotein B from the liver and lipid accumulation in the liver. Considering liver morphology, we can observe the big oval liver hepatocytes with many lipid droplets. The aberrant glycosylation of apo-B relies on the presence of additional *N*-acetylglucosamine (GlcNAc) attached to mannose. This modification is a result of the increased expression of the N-acetylglucosaminyltransferase III (GnT-III). The second reason of lipid accumulation is the ectopic expression of alpha1,6-fucosyltransferase in the liver and also in the kidney. Numerous, small vacuoles with lipid droplets can be observed in liver morphology. In fatty liver disease, we observed an increased fucosylation of lysosomal acid lipase, which caused a decreased activity of this enzyme (Blomme et al. 2009; Chrostek and Cylwik 2011). For this reason a significant increase in cholesterol esters and triglycerides was observed in the hepatocyte lysosomes.

The *N*-acetylglucosaminyltransferase III and *N*-acetylglucosaminyltransferase V (GnT-V) are responsible for altered glycosylation in viral liver diseases (Blomme et al. 2009; Chrostek and Cylwik 2011). Both GnT-III and GnT-V compete for the same substrate. This means that the increased activity of one of the *N*-acetylglucosaminyltransferases inhibits the expression of the second enzyme. The fetal hepatocyte cell line transfected with HBV showed an increased expression of *N*-acetylglucosaminyltransferase III. This is the reason of the presence of an extra *N*-acetylglucosamine attached to mannose (Shim et al. 2004). The HBV-transfected hepatocytes accumulate aberrant glycosylated apolipoprotein B, triglyceride, and cholesterol, which is a cause of the inhibition of the release of lipoproteins containing apo-B from the liver. In contrast to HBV-transfected fetal hepatocyte line, the hepatoblastoma cell line transfected with HBV showed a specific decrease in activity of GnT-III. The inhibition of GnT-III activity is a reason of increased GnT-V activity and creation of glycoprotein phenotype with highly branched N-glycans. Some reports suggest that the alpha1-acid glycoprotein could be a discriminating factor between viral liver diseases. The hyperfucosylation of this glycoprotein is present in all liver diseases, especially in both hepatitises B and C, but in patients with hepatitis C also occur, rarely presented in N-glycans, an additional N-acetylgalactosamine residue (Blomme et al. 2009).

The most characteristic change in the structure of N-glycans of glycoproteins in liver tumors is fucosylation of alpha-fetoprotein (AFP) and alpha1-antitrypsin (Saitoh et al. 1993; Blomme et al. 2009; Chrostek and Cylwik 2011). This structural modification is a result of increased activity of alpha1,6-fucosyltransferase. There are the fucosylated variants of alpha-fetoprotein named as AFP-L3, which are specifically recognized by specific lectin *Lens culinaris* agglutinin A. The increase of fucosylation index (ratio of AFP-L3 fraction to total alpha-fetoprotein) is a discriminating factor between patients with chronic nonmalignant liver diseases and liver cancer (Sterling et al. 2007). The increased levels of AFP-L3 variants indicate the poor prognosis and survival in patients with hepatocellular carcinoma (HCC), because this fraction is associated with large-size tumors, occupying the portal vein and with distant metastases. Although the fucosylation index of alpha1-antitrypsin is significantly higher in tumors than nonmalignant liver diseases, the fucosylated variants of alpha1-antitrypsin do not allow to differentiate liver cirrhosis from liver cancer (Saitoh et al. 1993). The second characteristic feature for liver cancer glycosylation is the formation of new antennas in the structure of AFP and AAT (Blomme et al. 2009; Chrostek and Cylwik 2011). Besides the fucosylated diantennary AFP forms, there are tri-, tetra-, and pentaantennary glycans. The increase in glycan branching is correlated with tumor invasiveness. The increased activity of GnT-V is positively correlated with the stage of development of tumor by TNM (tumor-node-metastases) staging classification. Moreover, in liver cancer we have also observed a double amount of *N*-acetylglucosamine attached to mannose, which is a result of increased activity of *N*-acetylglucosaminyltransferase III. In contrast to the chronic hepatitis and cirrhosis, in liver cancer the activity of GnT-III is clearly increased. It means that GnT-III may be a marker for differentiating precancerous conditions from liver cancer.

The Serum Concentration of Sialic Acid in Liver Diseases

For a better understanding of the changes occurring in sialic acid concentrations in the liver diseases, a normal range of sialic acid concentrations should be recalled. It is essential to know that the total sialic acid (TSA) is a sum of protein-bound sialic acid (PBSA), lipid-bound sialic acid (LSA), and free sialic acid (FSA). According to the literature data, the normal concentration of total sialic acid in human blood is between the range 51 and 84 mg/dL (Sillanaukee et al. 1999). The reference value for the lipid-bound sialic acid is 10–50 mmol/L, and for free sialic acid form, values are in the range of 0.5–3 μmol/L. The increased serum concentrations of sialic acid have been observed in different conditions, especially alcohol abuse, inflammatory diseases, malignancies, diabetes, and cardiovascular diseases (Kloppel et al. 1977; Stefenelli et al. 1985; Arif et al. 2005; Nigam et al. 2006; Varki 2008). In liver diseases, such as liver cirrhosis, liver cancer, fatty liver, and acute and chronic hepatitis, the variations in serum concentration of total sialic acid have been also described (Matsuzaki et al. 1981; Stefenelli et al. 1985; Cylwik et al. 2010; Chrostek et al. 2014; Gruszewska et al. 2014; Malik et al. 2015).

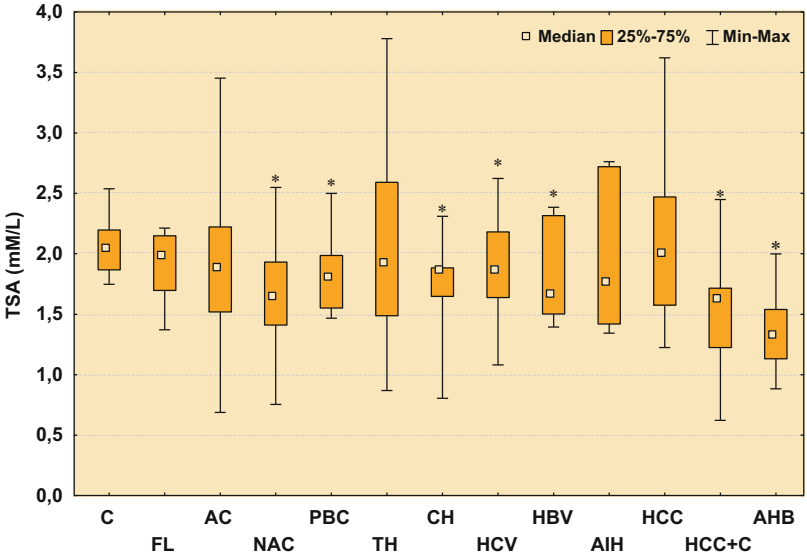

Fig. 4 TSA concentration in liver diseases. *FL* fatty liver, *AC* alcoholic cirrhosis, *NAC* nonalcoholic cirrhosis, *PBC* primary biliary cirrhosis, *TH toxic hepatitis*, *CH* chronic nonviral hepatitis, *HCV* chronic viral C hepatitis, *HBV* chronic viral B hepatitis, *AIH* autoimmune hepatitis, *HCC* primary liver cancer, *HCC+C* primary liver cancer and cirrhosis, *AHB* acute hepatitis B, *C* control group. * – $P < 0.05$ compared with C grou*p* (Gruszewska et al. 2014)

With accordance to the literature data, the concentration of total sialic acid in liver diseases is commonly lower than that in the healthy subjects. Previously data indicate that total sialic acid concentrations decreased in hepatitis of different etiology, in cirrhosis, and also in liver cancer (Fig. 4) (Gruszewska et al. 2014). Matsuzaki and co-workers (1981) have shown that the serum concentration of total sialic acid in patients with compensated cirrhosis is significantly lower in comparison to the control group. Moreover they have also indicated that TSA concentration decreased further in patients with decompensated cirrhosis. The level of TSA appeared to be similar in chronic hepatitis and in healthy subjects (Gruszewska et al. 2014). Interestingly, the same data also has stated that TSA concentrations in patients with alcoholic cirrhosis are similar with the concentration in control group. This fact may be explained by two opposing mechanisms. At first, the alcohol abuse increases TSA concentrations, and secondly, the cirrhosis causes the decrease of TSA levels. Stefenelli and co-workers (1985) have shown significantly decreased TSA levels in cirrhosis when compared to noninflammatory and malignant liver diseases. This could be the proof for the second mechanism presented. These results are in accordance with that obtained by Matsuzaki and co-workers (1981), who have also shown that serum TSA concentrations in patients with compensated cirrhosis were significantly lower than in the control group and decreased further in patients

with decompensation. In contrast to the above results, Arif and co-workers (2005) have shown higher concentrations of sialic acid in advanced and terminal stages of liver diseases, but normal levels in early ones. They explain that these results are related with changes in carbohydrate structure of fibrinogen, which contains 0.6% of sialic acid. Both components the fibrinogen and sialic acid are the acute-phase reactants.

It is well known that synthesis (also sialylation), degradation (desialylation), and storage of the lipids and lipoproteins take place in the liver. Because of this fact, it is possible to expect that the liver diseases affect not only the serum level of lipids and lipoproteins but also the level of sialic acid bounded with these compounds (LSA). There are significant data concerning increased levels of serum LSA in cancers of various organs. Also in liver cancer, there is the increased concentration of LSA. From previously reported data, there is evidence of concentrations of lipid-bound sialic acid in patients with liver cancer, liver cancer with cirrhosis, and toxic hepatitis (Fig. 5) (Chrostek et al. 2011). Moreover, there are data suggesting that besides the changes in the LSA concentrations in comparison to the control group, the LSA concentrations differ between liver diseases. For example, the level of LSA in primary liver cancers is higher than in alcoholic and nonalcoholic liver cirrhosis.

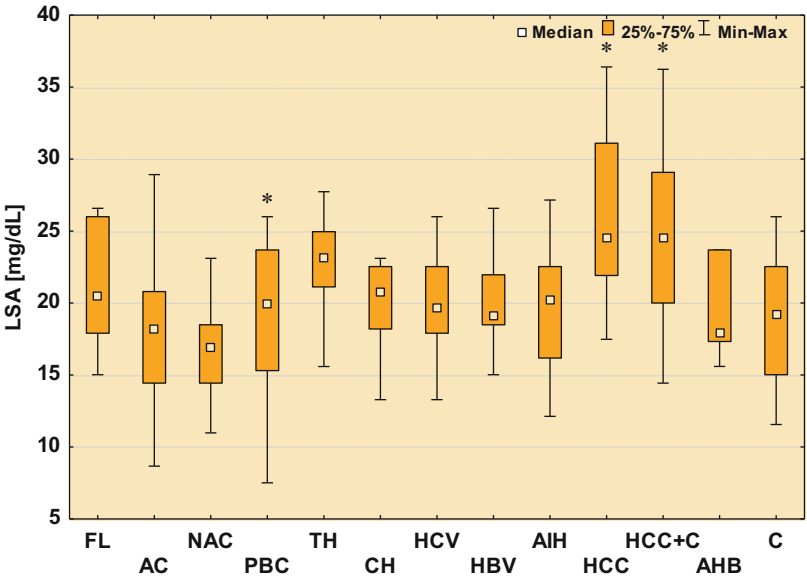

Fig. 5 LSA concentration in liver diseases. *FL* fatty liver, *AC* alcoholic cirrhosis, *NAC* nonalcoholic cirrhosis, *PBC* primary biliary cirrhosis, *TH* toxic hepatitis, *CH* chronic nonviral hepatitis, *HCV* chronic viral C hepatitis, *HBV* chronic viral B hepatitis, *AIH* autoimmune hepatitis, *HCC* primary liver cancer, *HCC+C* primary liver cancer and cirrhosis, *AHB* acute hepatitis B, *C* control group. * – $P < 0.05$ compared with C group (Chrostek et al. 2011)

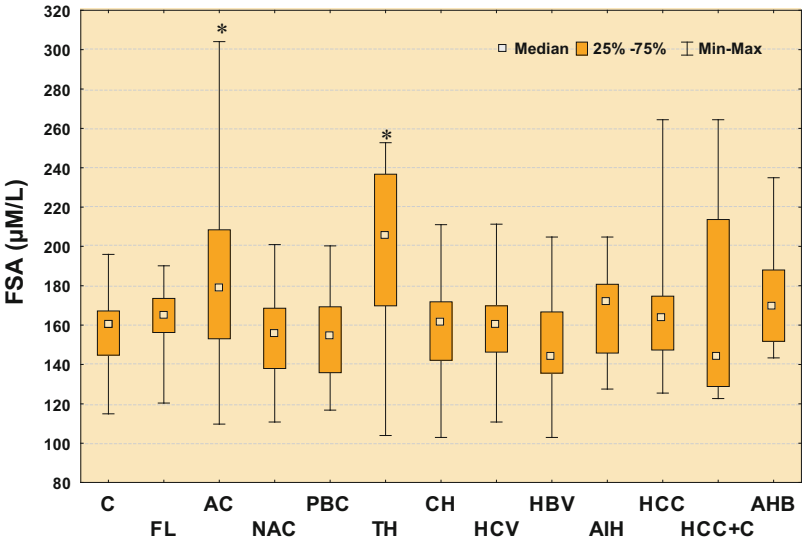

Fig. 6 FSA concentration in liver diseases. *FL* fatty liver, *AC* alcoholic cirrhosis, *NAC* nonalcoholic cirrhosis, *PBC primary biliary cirrhosis*, *TH* toxic hepatitis, *CH* chronic nonviral hepatitis, *HCV* chronic viral C hepatitis, *HBV* chronic viral B hepatitis, *AIH* autoimmune hepatitis, *HCC* primary liver cancer, *HCC+C* primary liver cancer and cirrhosis, *AHB* acute hepatitis B, *C* control group. * – $P < 0.05$ compared with C group (Gruszewska et al. 2014)

There are also data suggesting that LSA concentrations are higher in toxic hepatitis than in nonalcoholic cirrhosis (Chrostek et al. 2011). Matsuzaki and co-workers (1981) reported higher sialic acid level in hepatoma and metastatic liver cancer than in the controls and also higher than in the patients with cirrhosis.

It has been reported that FSA concentrations are significantly higher in toxic hepatitis than in nonalcoholic cirrhosis (Fig. 6) (Gruszewska et al. 2014). In these cases, the higher level of FSA concentration is the result of aberrant glycosylation in alcohol abusers. The proof for this argument may be the comparison of FSA levels in patients with alcoholic and nonalcoholic cirrhosis. While in nonalcoholic cirrhosis, the FSA concentration is the same as in the healthy subjects, the FSA concentration in alcoholic cirrhosis is significantly higher in comparison to the controls (Gruszewska et al. 2014). The FSA concentrations in patients with alcoholic cirrhosis are also higher than that in patients suffering from chronic viral hepatitis (Cylwik et al. 2010), while the serum concentrations of FSA in patients with nonalcoholic cirrhosis did not differ from patients with chronic viral hepatitis. The confirmation for thesis that alcohol abuse elevates the concentrations of sialic acid may be given by the study of Malik and co-workers (2015). The study has shown the significant increase of sialic acid concentration in alcoholic liver disease. They reported that protein-bound sialic acid levels are highly increased in ALD, which indicated that the cell damage occurred and the glycolipids or glycoproteins containing sialic acid were released into blood serum.

Pathomechanism of Changes in Sialylation and Sialic Acid Concentration

The variations in the sialic acid concentrations described above are related with different modifications of glycosylation. Due to the fact that the synthesis of most of the proteins and lipids and their glycosylation (also including sialylation) takes place in the liver, protein and lipid abnormal glycosylation plays an important role in the pathogenesis and progression in liver diseases (Traving and Schauer 1998; Blomme et al. 2009). From all serum proteins, only CRP and albumin are not glycosylated. One of the major reasons is the changes in the activity of the enzymes involved in the synthesis of glycans, such as glycosyltransferases and glycosidases. For example, in the literature there are evidences that decreased sialyltransferase activity is associated with chronic liver diseases (Dabelic et al. 2004). Dabelic and co-workers (2004) suggested that the chronic stress during chronic liver diseases is associated with decreased activity of sialyltransferases in the liver, in contrast to the acute stress, which increases activity of this enzyme in the liver. The increased concentration of sialic acid in malignancies can be explained by the changes in the metabolism of the tumor cell surface sialoglycoproteins and sialoglycolipids due to the activation of tumor characteristic enzymes – glycosyltransferases. For example, the activity of GnT-III in serum samples of hepatocellular carcinoma patients is significantly upregulated. The increased concentration of FSA may be stimulated by the higher release of sialic acid from surface glycoconjugates, as well as from the other hydrolyzed structures – mucins (Beatty et al. 2001; Gruszewska et al. 2013). There are reports suggesting that sialidases, the enzymes which catalyze the removal of sialic acid residues from the glycoconjugates, are responsible for the altered sialylation of cell surface glycoproteins and glycolipids (Miyagi et al. 2008). For example, Miyagi and co-workers have shown that the sialidase present in the plasma membrane of tumor cell showed a marked upregulation.

As was mentioned earlier, the synthesis, degradation, storage, and glycosylation of lipids and lipoproteins are attributed to the liver. So, the liver diseases may affect the serum concentrations of sialic acid bounded with these compounds. Therefore, the changes in the lipid-bound sialic acid concentrations may be explained by the alterations in the metabolism of cell surface sialoglycoproteins and sialoglycolipids (Varki 2008). During the malignant transformations, these compounds on the tumor cell surface, especially sialoglycolipids (glycoconjugates), are released into the blood serum (Kloppel et al. 1977). This secretion and/or shedding from malignant cells leads to an elevation of sialic acid levels in blood. Moreover Barkal and Di Cesare (1975) have shown that glycosphingolipids containing sialic acid (i.e., gangliosides) have relatively long retention times in the blood in comparison to asialolipids (neutral glycolipids). Therefore, this data together with the reports about shedding of tumor antigens and membranes from the tumor cells suggested that gangliosides might be accumulated in the serum.

Another mechanism of changes in sialic acid concentrations may be the release of acute-phase proteins from the liver as a response of the body for the existing inflammatory process (Narayanan 1994; Sillanaukee et al. 1999). The literature data suggested that in this phenomenon participates interleukin-1 (IL-1), which is

released from macrophages during inflammation. This interleukin stimulates the synthesis of acute-phase proteins in the liver. It leads to an increase of serum acute-phase proteins containing sialic acid, such as fibrinogen, haptoglobin, ceruloplasmin, alpha1-acid glycoprotein, alpha1-antitrypsin, and alpha2-macroglobulin (Nigam et al. 2006). The increased serum concentrations of these proteins may increase serum concentration of total sialic acid (Sillanaukee et al. 1999). This may be proved be the fact that TSA concentrations correlate positively with pro-inflammatory cytokines, e.g., tumor necrotic factor alpha (TNF-α) and interleukin-6 (IL-6), which are responsible for the regulation of the synthesis of C-reactive protein and other acute-phase proteins (Sillanaukee et al. 1999). Some literature data suggest that simultaneous determination of TSA and LSA concentrations may be useful in the differentiation of inflammatory processes (elevated TSA with normal LSA concentrations) from malignant transformation (elevated TSA and LSA concentrations) (Stefenelli et al. 1985).

Diagnostic Usefulness of Aberrant Glycosylation in Liver Diseases

The changes in glycosylation described above lead to the presence of aberrant glycan profiles characteristic for different liver diseases. Due to this fact, the multiple tests based on glycan structure of serum glycoproteins have been developed, such as GlycoFibro test, GlycoCirrho test, and GlycoHCC test (Blomme et al. 2009; Chrostek and Cylwik 2011). The GlycoFibro test, used for monitoring liver fibrosis, is the ratio of diantennary agalacto glycans with extra *N*-acetylglucosamine to the fully galactosylated triantennary glycans. The GlycoCirrho test, used for detection of liver cirrhosis, calculates the ratio between the diantennary agalacto glycans with core fucose and fully galactosylated triantennary glycans. This test has a good diagnostic value (75% sensitivity and 100% specificity) to distinguish compensated cirrhosis from non-cirrhotic chronic liver diseases. In turn, the GlycoHCC test uses the logarithmic ratio of a triantennary galactosylated glycans with distant fucose to diantennary galactosylated glycans with extra *N*-acetylglucosamine and core fucose, similar to alpha-fetoprotein sensitivity and specificity in differentiating hepatocellular cancer from cirrhosis.

Potential Applications to Prognosis, Other Diseases, or Conditions

The changes in serum sialic acid concentration are reported in many various diseases, but its major diagnostic usefulness is in tumor diseases, which have an impact on some biological functions of sialic acid. There are reports that indicate an increased serum level of total and lipid-bound sialic acid in various types of cancers (e.g., ovarian, oral, lung, cervical, rectal, stomach, breast, and many others). Moreover, the serum concentrations of sialic acid have been observed to correlate positively with cancer advancement.

Furthermore, sialic acid appears to be interesting biochemical marker of excessive alcohol consumption. There are reports that indicate that the sialic acid measurement can be a valuable marker both for detecting and monitoring alcohol abuse. The clinical studies showed that serum sialic acid concentrations were significantly higher in alcohol abusers (both in males and in females) than that in the social drinkers. Diagnostic efficiency of sialic acid is as good as or even better than the traditional markers of alcohol abuse, e.g., CDT, GGT, AST, and ALT. The generally observed nonspecificity of sialic acid to a certain disease could limit the clinical usefulness of its determination in alcoholics. But this could be eliminated when sialic acid measurements were combined with determination of another biomarkers of alcohol abuse.

Summary Points

- *N*-Acetylneuraminic acid is the most common sialic acid in human.
- The differences in serum concentrations of sialic acid have been reported in many diseases.
- The serum level of sialic acid especially differs between liver diseases.
- The changed concentrations of all forms of sialic acid (protein bound, lipid bound, and free form) during liver diseases are the result of alterations in the sialylation of proteins and lipids.
- The changes in sialic acid concentrations are characteristic for the liver diseases of different etiologies and can affect the pathogenesis and progression of liver disease.
- The estimation of serum sialic acid concentration may be helpful in the diagnosis and monitoring of treatment of liver diseases of different etiology.

References

Arif S, Haq N, Hanif R, Khan AS, Rehman J, Mufti TA. Variations of serum sialic acid levels in liver cirrhosis. J Ayub Med Coll Abbottabad. 2005;17(3):54–7.

Barkal A, Di Cesare JL. Influence of sialic acid groups on the retention of glycosphingolipids in blood plasma. Biochim Biophys Acta. 1975;398(2):287–93.

Beatty P, Hanisch FG, Stolz DB, Finn OJ, Ciborowski P. Biochemical characterization of the soluble form of tumor antigen MUC1 isolated from sera and ascites fluid of breast and pancreatic cancer patients. Clin Cancer Res. 2001;7(3):781–7.

Blomme B, Steenkiste C, Callewaert N, Vlierberghe H. Alteration of protein glycosylation in liver diseases. J Hepatol. 2009;50(3):592–603.

Bork K, Horstkorte R, Weidemann W. Increasing the sialylation of therapeutic glycoproteins: the potential of the sialic acid biosynthetic pathway. J Pharm Sci. 2009;98(10):3499–508.

Chrostek L, Cylwik B. Zaburzenia glikozylacji w chorobach wątroby. Pol Merkuriusz Lek. 2011; 31(181):60–4.

Chrostek L, Cylwik B, Szmitkowski M, Korcz W. The diagnostic accuracy of carbohydrate-deficient transferrin, sialic acid and commonly used markers of alcohol abuse during abstinence. Clin Chim Acta. 2006;364(1-2):167–71.

Chrostek L, Cylwik B, Panasiuk A, Brodowska-Adamusiak D, Gruszewska E. Lipid-bound sialic acid (LSA) in liver diseases of different etiologies. Ann Hepatol. 2011;10(2):150–4.
Chrostek L, Cylwik B, Gindzienska-Sieskiewicz E, Gruszewska E, Szmitkowski M, Sierakowski S. Sialic acid level reflects the disturbances of glycosylation and acute-phase reaction in rheumatic diseases. Rheumatol Int. 2014;34(3):393–9.
Cylwik B, Chrostek L, Panasiuk A, Szmitkowski M. Serum total and free sialic acid in patients with chronic liver disease. Clin Chem Lab Med. 2010;48(1):137–9.
Dabelic S, Flögel M, Maravić G, Lauc G. Stress causes tissue-specific changes in the sialyltransferase activity. Z Naturforsch C. 2004;59(3-4):276–80.
Ghosh P, Hale EA, Lakshman MR. Plasma sialic acid index of apolipoprotein J (SIJ): a new alcohol intake marker. Alcohol. 2001;25(3):173–9.
Gruszewska E, Chrostek L, Cylwik B, Tobolczyk J, Szmitowski M, Kukliński A, Kedra B. Serum sialic acid as a marker of pancreatic cancers. Clin Lab. 2013;59(7-8):781–8.
Gruszewska E, Cylwik B, Panasiuk A, Szmitkowski M, Flisiak R and Chrostek L. Total and free serum sialic acid concentration in liver diseases. BioMed Res Int. [online] 2014, article ID 876096. Available from: http://dx.doi.org/10.1155/2014/876096.
Kloppel TM, Keenan TW, Freeman MJ, Morre DJ. Glycolipid-bound sialic acid in serum: increased levels in mice and humans bearing mammary carcinomas. Proc Natl Acad Sci U S A. 1977; 74(7):3011–3.
Kongtawelert P, Tangkijvanich P, Ong-Chai S, Poovorawan Y. Role of serum total sialic acid in differentiating cholangiocarcinoma from hepatocellular carcinoma. World J Gastroenterol. 2003;9(10):2178–81.
Lakshman MR, Rao MN, Marmillot P. Alcohol and molecular regulation of protein glycosylation and function. Alcohol. 1999;19(3):239–47.
Li Y, Chen X. Sialic acid metabolism and sialyltransferases: natural functions and applications. Appl Microbiol Biotechnol. 2012;94(4):887–905.
Malik K, Chugh K, Gupta G, Dahyia K, Gulia D, Tiwari R. Significance of protein bound sialic acid in alcoholic liver disease. IJIMS. 2015;2(6):8–12.
Matsuzaki S, Itakura M, Iwamura K, Kamiguchi H. Serum sialic acid levels in liver cirrhosis and liver cancer. Gastroenterol Jpn. 1981;78(12):2395–401.
Miyagi T, Wada T, Yamaguchi K, Shiozaki K, Sato I, Kakagawa Y, Yamanami H, Fujiya T. Human sialidase as a cancer marker. Proteomics. 2008;8(16):3303–11.
Nakagawa T, Uozumi N, Nakano M, Mizuno-Horikawa Y, Okuyama N, Taguchi T, Gu J, Kondo A, Taniguchi N, Miyoshi E. Fucosylation of N-glycans regulates the secretion of hepatic glycoproteins into bile ducts. J Biol Chem. 2006;281(40):29797–806.
Narayanan S. Sialic acid as a tumor marker. Ann Clin Lab Sci. 1994;24(4):376–84.
Nigam PK, Narain VS, Kumar A. Sialic acid in cardiovascular diseases. Indian J Clin Biochem. 2006;21(1):54–61.
Saitoh A, Aoyagi Y, Asakura H. Structural analysis on the sugar chains of human alpha1-antitrypsin: presence of fucosylated biantennary glycan in hepatocellular carcinoma. Arch Biochem Biophys. 1993;303(2):281–7.
Schauer R, Kamerling JP. Chemistry biochemistry and biology of sialic acids. In: Montreuil J, Vliegenthart JFG, Schachter H, editors. Glycoproteins II. Amsterdam: Elsevier; 1997. p. 243–402.
Shim JK, Lee YC, Chung TH, Kim CH. Elevated expression of bisecting *N*-acetylglucosaminyltransferase-III gene in a human fetal hepatocyte cell line by hepatitis B virus. J Gastroenterol Hepatol. 2004;19(12):1374–87.
Sillanaukee P, Pönniö M, Jääskeläinen IP. Occurrence of sialic acid in healthy humans and different disorders. Eur J Clin Invest. 1999;29(5):413–25.
Stefenelli N, Klotz H, Engel A, Bauer P. Serum sialic acid in malignant tumors, bacterial infections, and chronic liver diseases. J Cancer Res Clin Oncol. 1985;109(1):55–9.
Sterling RK, Jeffers L, Gordon F, Sherman M, Venook AP, Reddy KR, Satomura S, Schwartz ME. Clinical utility of AFP-L3% measurement in North American patients with HCV-related cirrhosis. Am J Gastroenterol. 2007;102(10):2196–205.

Traving C, Schauer R. Structure, function and metabolism of sialic acids. Cell Mol Life Sci. 1998;54(12):1330–49.

Tsutsumi M, Takase S. Usefulness of microheterogeneity of serum alpha 1-acidglycoprotein as a marker for alcohol abuse. Alcohol. 2001;25(3):181–4.

Varki A. Sialic acids in human health and disease. Trends Mol Med. 2008;14(8):351–60.

Waters PJ, Lewry E, Peunock CA. Measurement of sialic acid in serum and urine: clinical applications and limitations. Ann Clin Biochem. 1992;29(6):625–37.

Osteopontin as a Biomarker in Liver Disease 20

Radan Bruha

Contents

Abstract

Osteopontin (OPN) is a multifunctional protein, physiologically expressed in the kidney and bone. OPN expression has been attributed to many pathological conditions including inflammation, angiogenesis, fibrosis, and carcinogenesis. OPN was found to contribute to the migration of macrophages into necrotic areas in liver tissue and to serve as a key cytokine within the extracellular matrix in the liver, contributing to fibrogenesis. OPN is also involved in the evolution and progression of various cancers, including hepatocellular carcinoma. Plasma OPN levels were found to predict liver fibrosis in various chronic liver diseases such as nonalcoholic steatohepatitis, alcoholic liver disease, and chronic viral hepatitis. OPN levels correlate significantly with the degree of fibrosis and could serve as a

R. Bruha (✉)
General University Hospital, Charles University in Prague, Prague 2, Czech Republic
e-mail: bruha@cesnet.cz

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_44

noninvasive biomarker of portal hypertension and as a prognostic parameter in cirrhosis.

Keywords

Alcoholic liver disease • Cirrhosis • Liver fibrosis • Nonalcoholic liver disease • Osteopontin • Portal hypertension

List of Abbreviations

ALT	Alaninaminotransferase
AST	Aspartataminotransferase
HCC	Hepatocellular carcinoma
HMGB1	High-mobility group box-1
HSC	Hepatic stellate cell
HVPG	Hepatic venous pressure gradient
iNOS	Inducible nitric oxide synthase
NASH	Nonalcoholic steatohepatitis
NO	Nitric oxide
OPN	Osteopontin

Key Facts of Liver Fibrosis and Cirrhosis

- Liver fibrosis is a response of liver parenchyma to chronic inflammatory processes.
- Fibrosis could lead to liver cirrhosis with its high morbidity and mortality.
- Liver fibrosis and to a certain degree also cirrhosis are reversible.
- The main factors leading to chronic liver disease worldwide are nonalcoholic fatty liver disease, alcoholic liver disease, and chronic viral hepatitis.
- Osteopontin is one of the cytokines involved in fibrogenesis.
- Osteopontin targeting could have therapeutical consequences in the future.

Definitions of Words and Terms

Cirrhosis	A final stage of different chronic liver diseases. It is responsible for high morbidity and mortality in patients with advanced liver disease.
Hedgehog pathway	This is a morphogen important for embryonic development. It is involved in different pathological processes in adults.
Hepatic stellate cells (HSCs)	HSCs are non-parenchymal liver cells, which can be under pathological processes transformed to the proliferative

	myofibroblasts responsible for secreting extracellular matrix and liver fibrosis.
Hepatic venous pressure gradient (HVPG)	Invasive measurement of portal hypertension. It needs catheterisation of liver veins (via the jugular or femoral vein). Until now no noninvasive test could fully substitute HVPG measurement.
High-mobility group box-1 (HMGB1)	HMGB1 is a nuclear nonhistone chromosomal protein that binds the DNA minor groove and is involved in DNA replication, repair, and energy homeostasis.
Liver fibrosis	The net accumulation of extracellular matrix (scarring) in liver tissue. Fibrosis results from many pathological stimuli, and it is a dynamic and bidirectional process, involving phases of progression and regression. Increased distortion of the normal liver architecture results in cirrhosis.
Nonalcoholic fatty liver disease (NAFLD)	Represents the hepatic manifestation of a metabolic syndrome: a disease affecting a substantial portion of modern populations. NAFLD comprises a spectrum of conditions from simple steatosis, through nonalcoholic steatohepatitis (NASH), and ending with liver cirrhosis. With the epidemic increase in the incidence of obesity, NAFLD has become a serious issue for the future.
Portal hypertension	Increased pressure in the portal system, a consequence of cirrhosis. Portal hypertension accounts for the majority of fatal complications of liver cirrhosis such as bleeding from oesophageal varices, ascites, and hepatic encephalopathy.

Introduction

Osteopontin (OPN), first described in 1979 (Senger et al. 1979), is a multifunctional protein physiologically expressed mainly in the kidney and bone tissue (Oldberg et al. 1986). Under pathological conditions, OPN expression has been found in various organs and attributed to many pathological conditions including inflammation, angiogenesis, fibrosis, and carcinogenesis (Nagoshi 2014). OPN enhances the migration of macrophages into necrotic areas in the liver tissue (Ramaiah and

Rittling 2008) and serves as a key cytokine within the extracellular matrix, thus adding to fibrogenesis (Urtasun et al. 2012; Leung et al. 2013). OPN is involved in the evolution and progression of various cancers, including hepatocellular carcinoma (HCC) (Gotoh et al. 2002) and cholangiocarcinoma (Terashi et al. 2004).

Structure and Function

OPN belongs to the small integrin-binding N-linked glycoprotein family (SIBLING) (Fisher et al. 2001), which is expressed by a gene located at the long arm of chromosome 4 (4q13) as a ~34-KDa protein (Young et al. 1990). Structurally, OPN is roughly globular, with distinctive features (Table 1): It contains a poly-Asp sequence which is involved in binding the protein to the hydroxyapatite matrix of bone (Sorensen et al. 1995) and an RGD sequence (arginine-glycine-aspartate) which mediates interaction with integrins (mainly those of the α_v class: $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_1$) (Liaw et al. 1995). In addition, OPN was found to bind to non-integrin cell surface receptor CD-44 (Weber et al. 1996) (Fig. 1). OPN is secreted through the endoplasmic reticulum and Golgi as a "sOPN" isoform to the extracellular space allowing autocrine and paracrine signaling (Kazanecki et al. 2007; Uede 2011). Another isoform may remain inside the cell –"iOPN" (Zohar et al. 1997).

OPN was first discovered as a main sialoprotein of the extracellular matrix of bone tissue (Franzen and Heinegard 1985). Its name reflects the potential to serve as a "bridge" between cells and hydroxyapatite in the bone matrix (Oldberg et al. 1986). Other functions associated with cell signaling and potentially involved in different pathological processes were described later (Table 2). OPN is secreted by activated

Table 1 Binding sites of osteopontin and their function

Osteopontin structure	Function – target site for binding
Poly-Asp sequence	hydroxyapatite matrix of bone
RGD sequence and SVVYGLR domain	integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_1$)
Non–integrin-binding domains	CD44

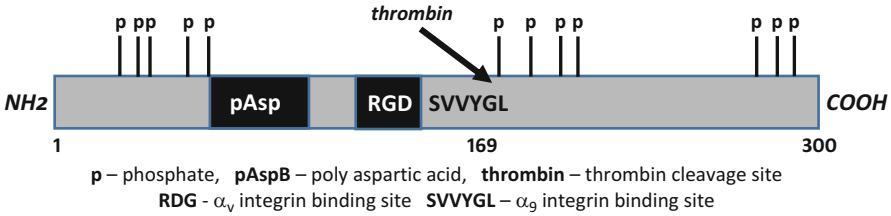

Fig. 1 Structure of osteopontin. Domain structure of osteopontin showing 13 phosphorylation sites based on sites identified in bovine bone (Sorensen and Petersen 1994). Polyaspartic acid-containing domain pAsp, RGD- containing α_v integrin adhesive domain, SVVYGL-containing $\alpha_9\beta_1$ binding site, and thrombin cleavage site. Numbers refer to amino acid positions

Table 2 The role of osteopontin in different organ disease

Organ/disease	Role of osteopontin
Autoimmune diseases	Upregulation; mediator in pathogenesis
Brain neuroinflammatory diseases	Upregulation; positive role, activation of neural stem cells
Kidney	Divergent role, not fully understood
Atherosclerosis	Upregulation; mediator in pathogenesis – inhibition of iNOS
Cancer	Upregulation; role in migration or adhesion of tumour cells

macrophages and T-lymphocytes, causing macrophages to migrate and enhancing immunoglobulin generation and B-lymphocyte proliferation. The secreted isoform of OPN is involved in the generation of T helper type 1 (Th1) and Th17 cells, i.e., pathogenic T cells for various autoimmune diseases. In fact, a critical role of OPN in type T cell-mediated immunity has been demonstrated, in which OPN increases IL-2, while suppressing IL-10 (Ashkar et al. 2000). OPN, therefore, might be instrumental in the development of different autoimmune diseases such as lupus erythematosus (D'Alfonso et al. 2005), type I diabetes mellitus (Karamizadeh et al. 2013), and Sjögren's syndrome (Husain-Krautter et al. 2015). Indeed, OPN-deficient mice are resistant to various Th1-related autoimmune diseases (Uede 2011).

OPN is expressed constitutively also in the brain and upregulated during neuroinflammatory responses (Hedtjarn et al. 2004). Rabenstein et al. (2015) described positive OPN effects on the survival, proliferation, migration, and neuronal differentiation of neural stem cells. These effects were mediated partly via the CXC chemokine receptor type 4 (CXCR4). The above authors of that paper suggested that OPN might be a promising substance for targeted activation of neural stem cells in future experimental therapies for neurological disorders such as stroke.

As OPN possess several adhesive domains, which interact with cell surface integrins and CD44 surface receptor, it could take part in the migration or adhesion of tumor cells (Wai and Kuo 2004) and link OPN to tumor growth and progression (Sodek et al. 2000). OPN has been detected in many tumors and for example proposed as a specific marker for breast cancer (Mirza et al. 2008).

OPN was also found in atherosclerotic lesions in association with foam cells and macrophages and hypothesized to play a role in the development and progression of atherosclerosis (Isoda et al. 2003). The relation of OPN to atherosclerosis development could be attributed to another OPN activity: it inhibits the induction of nitric oxide synthase (iNOS) by inflammatory mediators (Hwang et al. 1994).

OPN has a not fully understood part to play in the kidney. It is involved in the kidney evolution in vitro (Rogers et al. 1997), but in the OPN-deficient mice, kidney development is entirely normal, without any histological abnormalities (Rittling and Denhardt 1999). OPN appears to have a diverse and multiple roles mainly in pathological renal processes, as described in OPN-deficient mice (Noiri et al. 1999). In some cases (such as in renal ischemia), tissue injury is greater in the absence of OPN, while in other cases (such as tumorigenesis), the absence of OPN is beneficial. Hence the pathological role of OPN is ambivalent and not yet fully understood.

Osteopontin in the Liver

Hepatic expression of OPN was first reported by Kawashima et al. (1999) in rats after carbon tetrachloride intoxication. The authors found minimal OPN mRNA expression in Kupffer cells and hepatocytes from normal rats. After liver necrosis induction by carbon tetrachloride administration, OPN mRNA expression was increased in the rat liver with its peak at 2 days following the intoxication. In particular, increased OPN mRNA expression was detected in Kupffer cells, hepatic macrophages, and hepatic stellate cells isolated from necrotic rat liver. Also immunohistochemically, OPN was detected in necrotic liver areas within the same cells: macrophages, Kupffer cells, and stellate cells. Moreover, the last mentioned authors used recombinant human OPN to prove a dose-related manner of augmentation to the migration of Kupffer cells triggered by OPN in vitro. In an experimental model of liver fibrosis, OPN was shown to serve as a key cytokine within the extracellular matrix protein network, responsible for scarring and liver fibrosis (Urtasun et al. 2012). OPN also delays liver fibrosis resolution due to sustained fibrillar collagen-I deposition in mice after thioacetamide-induced fibrosis (Leung et al. 2013). OPN upregulation during liver injury is a conserved repair response, acting on liver progenitor cell function. OPN-neutralization abrogates the liver progenitor cell response and fibrogenesis in murine models of liver fibrosis (Coombes et al. 2015). Transgenic mice overexpressing OPN in hepatocytes give rise to spontaneous liver fibrosis even in the absence of profibrogenic factors (Urtasun et al. 2012).

OPN acts as paracrine and autocrine signals to cause scarring in liver tissue (Arriazu et al. 2016) through the regulation of "High-mobility group box-1" (HMGB1; this is a nuclear nonhistone chromosomal protein that binds the DNA minor groove and is involved in DNA replication, repair, and energy homeostasis). OPN is upstream of HMGB1 and promotes the acetylation of intracellular HMGB1 in hepatic stellate cells (HSCs) due to increased NADPH oxidase activity and the associated decrease in histone deacetylases 1,2. It leads to upregulation of collagen-I.

Of late, plasma OPN levels were found to predict liver fibrosis in various chronic liver diseases such as nonalcoholic steatohepatitis (NASH) (Syn et al. 2012), alcoholic liver disease (Patouraux et al. 2012), and chronic viral hepatitis B (Zhao et al. 2008) and C (Huang et al. 2010) (Table 3).

Nevertheless, the role of OPN in liver injury seems to be not so obviously negative. Patouraux et al. (2014) showed that hepatic warm ischemia followed by reperfusion induced the upregulation of the hepatic and systemic level of OPN in mice. However, significantly higher incidence of hepatocyte necrosis and higher AST and ALT levels were found in OPN-deficient mice than in wild type mice. In addition, the expression levels of iNOS and TNFα were upregulated in OPN-deficient mice versus wild type mice. The authors explain this finding by decreased cell viability in OPN deficiency or by enhanced iNOS expression and sensitizing macrophages to inflammatory signals in OPN deficient animals. They conclude that OPN partially protects from hepatic injury and inflammation induced

Table 3 The role of osteopontin in liver diseases

Liver disease	The possible role of osteopontin
Fibrosis	Upregulation; mediator in pathogenesis (activation of HSC and liver progenitor cells)
Alcoholic liver disease	Upregulation; mediator of hepatic natural killer cells and neutrophil infiltration
Alcoholic liver disease	Binding to lipopolysaccharides, lowering of TNFα levels– prevention of early alcoholic hepatitis
CCl_4 intoxication	Upregulation; activation of hepatic macrophages and Kupffer cells
NASH	Upregulation; activation of hedgehog pathway
Chronic viral hepatitis	Biomarker of the degree of fibrosis
Cirrhosis	Biomarker of the degree of portal hypertension; parameter of liver dysfunction; prognostic parameter
HCC	Upregulation; prognostic parameter

in the experimental model of liver ischemic-reperfusion injury. This could be due to its ability to partially prevent hepatocyte death and to limit the production of toxic iNOS-derived NO by macrophages. Similarly, a protective effect of OPN against cardiac and renal ischemic injury was also reported in literature (Wang et al. 2009).

OPN in NAFLD/NASH

The increased hepatic expression of OPN was shown to correlate strongly with liver steatosis and insulin resistance in obese patients and mice in a study by Bertola et al. (2009). Increased OPN expression could be due to the accumulation of triglycerides, since fat deposition in cultured HepG2 cells promoted OPN expression.

The role of OPN in NASH was studied by Syn et al. (2010). They showed that NASH-related cirrhosis is associated with the activation of the Hedgehog pathway (a morphogen important for embryonic development). Such activation leads to the induction of liver progenitor cells causing them to proliferate, undergo epithelial mesenchymal transition, and secrete factors that activate neighbouring progenitors and matrix-producing cells. Osteopontin (OPN) is one such factor produced by progenitor cells. It enhances the development and progression of NASH in mice and humans. Recently, the same authors (Syn et al. 2011) reported that OPN is Hedgehog regulated and that it directly promotes profibrogenic responses both in vivo and in vitro experiments. They used methionine and choline-deficient diet to induce NASH-related fibrosis in mice. Animals with overly active Hedgehog signaling expressed more OPN and developed worse liver fibrosis than WT mice, whereas OPN-deficient mice had reduced fibrosis. In cultured hepatic stellate cells, Hedgehog agonists upregulated OPN expression, whereas Hedgehog antagonists repressed OPN expression. Fibrogenic responses in hepatic stellate cells were promoted by recombinant OPN, whereas attenuated by OPN neutralization. In NASH patients, OPN was significantly upregulated and correlated with Hedgehog

pathway activity and stage of fibrosis. The authors conclude that OPN induction correlates with Hedgehog pathway activity and stage of fibrosis and that OPN reflects the activity of fibrogenic processes in the liver. Therefore, first, OPN may prove to be a useful noninvasive marker of advanced NASH and, second, OPN inhibition may be beneficial in NASH.

OPN in Alcoholic Liver Disease

The divergent role of OPN in pathological processes appears in a great many of studies of alcoholic liver disease. Morales-Ibanez et al. (2013) investigated the pathogenic role of OPN in alcoholic hepatitis. They evaluated the role of OPN in patients with alcoholic hepatitis, as well as in murine model of alcoholic hepatitis. Hepatic expression and serum levels of OPN were markedly increased in patients with alcoholic hepatitis, compared to normal livers and other types of chronic liver disease, and correlated inversely with short-term survival. Serum levels of OPN also correlated with hepatic expression and disease severity. OPN was mainly expressed in areas with inflammation and fibrosis. Interestingly, OPN(−/−) mice were protected against alcohol-induced liver injury and showed decreased expression of inflammatory cytokines. The authors conclude that OPN hepatic gene expression paralleled with disease severity in patients with well-characterized alcoholic hepatitis and, second, that mice lacking OPN are resistant to ethanol-induced liver damage (Morales-Ibanez et al. 2013).

The pathogenic role of OPN for the development of alcoholic liver disease was described by Apte et al. (2005). Using a rat model of alcoholic steatosis and steatohepatitis, they described OPN-driven hepatic neutrophil infiltration. Taken together, their data indicate that OPN expression induced during alcoholic liver injury may play a significant role in the pathogenesis of alcoholic steatohepatitis throughout the stimulation of neutrophil transmigration.

Ge et al. (2014) used wild-type OPN(−/−) and transgenic mice overexpressing OPN in hepatocytes to investigate the role of OPN in alcohol-induced liver injury. They found that parameters of liver injury (steatosis, hepatocyte ballooning degeneration, as well as serum ALT activity) were lower in ethanol-treated transgenic mice overexpressing OPN than in OPN(−/−) mice. They also documented that OPN showed binding affinity for lipopolysaccharides whereas treatment with OPN blocked lipopolysaccharides translocation in vivo and protected from early alcohol-induced liver injury in mice. They concluded that natural induction plus forced overexpression of OPN in the liver or treatment with OPN protect from early alcohol-induced liver injury by blocking the gut-derived lipopolysaccharides and TNFα effects in the liver. The controversial role of OPN in alcoholic liver disease could be explained by the protective role of OPN for intestinal mucosa (Da Silva et al. 2006) rather than by OPN activity in the liver. In pathological processes other than alcoholic disease, OPN seems to play an undoubtedly negative role.

OPN in Viral Hepatitis

Matsue et al. correlated OPN levels with the degree of liver fibrosis in 115 patients with chronic HCV infection. The grading of liver fibrosis was based on liver biopsy. The authors found that OPN concentration significantly correlated with the degree of fibrosis and suggested that OPN could be used as a biomarker for the degree of liver fibrosis and for the prediction of disease progression (Matsue et al. 2015).

Also in patients with chronic HBV infection, plasma OPN level was found to serve as a predictor of cirrhosis (Zhao et al. 2008).

OPN in Cirrhosis and Portal Hypertension

As OPN levels correlate significantly with the stage of fibrosis in alcohol-induced liver disease (Patouraux et al. 2012), it might be assumed that OPN levels may be related to the degree of portal hypertension and hence serve as a surrogate noninvasive marker of portal hypertension and liver insufficiency (Fig. 2). We found that OPN plasma levels correlate strongly with the degree of portal hypertension in a cohort of 154 patients with liver cirrhosis (Bruha et al. 2016) (Fig. 3). Based on our results, OPN levels could be used in the selection of patients with clinically significant portal hypertension, i.e., those with hepatic venous pressure gradient (HVPG) above 10 mmHg. Moreover, OPN has been found to act as a strong prognostic indicator with respect to overall survival (Fig. 4; Table 4).

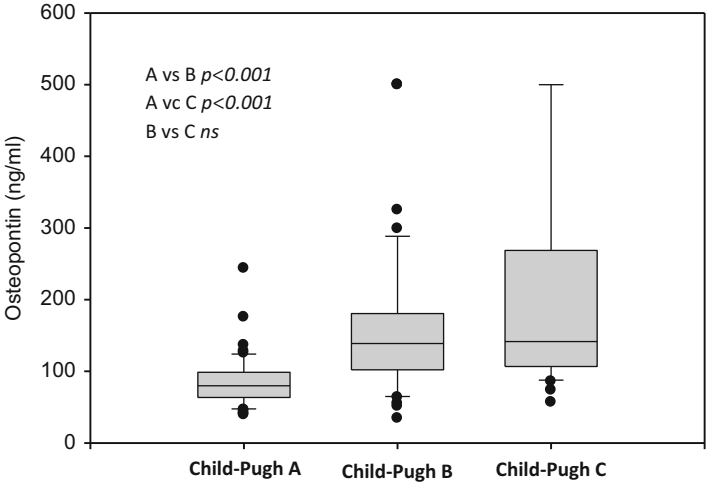

Fig. 2 Plasma OPN in patients with cirrhosis in Child-Pugh A, B and C classes (Bruha et al. 2016). *Box* – plot graphs; boxes correspond to the median value and interquartile range

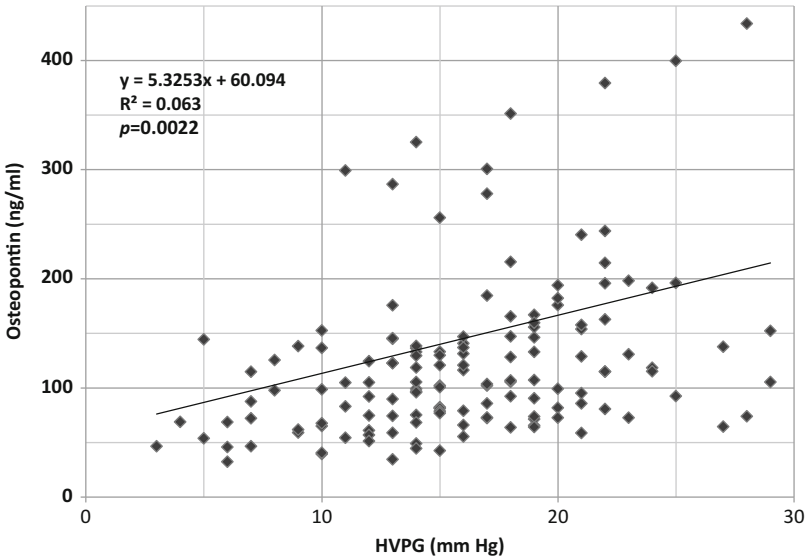

Fig. 3 Relationship between HVPG and plasma OPN concentrations in patients with cirrhosis (Bruha et al. 2016)

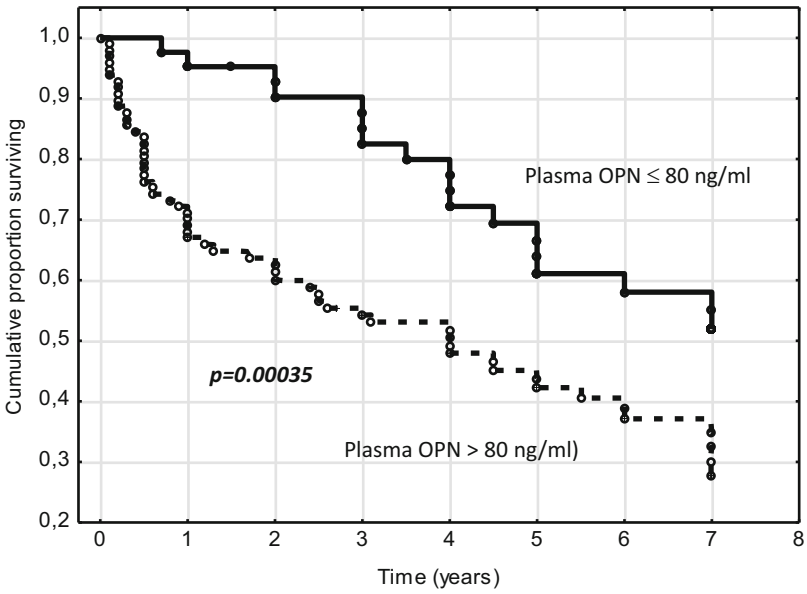

Fig. 4 Cumulative proportion of surviving patients with plasma OPN levels below and above 80 ng/ml in the group of 154 patients with liver cirrhosis. Kaplan-Meier method, the mean follow-up 3.7 ± 2.6 years (Bruha et al. 2016)

Table 4 Risk for death of patients with liver cirrhosis based on the cut-off values of HVPG and OPN

Parameter	Odds ratio	95% CI	*p*-value
HVPG > 10 mmHg	2.92	1.09–7.76	0.032
OPN > 80 ng/ml	2.23	1.06–4.68	0.034
[a]HVPG or OPN above cut-off	2.34	1.16–4.75	0.018
[b]Both HVPG and OPN above cut-off	5.09	1.29–20.15	0.02

[a]Only one parameter above cut-off values (10 mmHg for HVPG, 80 ng/l for OPN)
[b]Both parameters above cut-off (Bruha et al. 2016)

OPN in Hepatocellular Carcinoma

Using proteomic profiling of plasma from patients with cirrhosis and HCC, Shang et al. (2012) found OPN to be significantly upregulated in HCC cases, when compared to cirrhosis controls. Subsequently, plasma concentrations of OPN measured in cirrhotic patients, with and without HCC, revealed significantly higher concentrations in individuals with HCC than in those without tumours. Recently, Nabih et al. (2014) identified OPN as a tumour marker, which could be used as a screening test for the diagnosis of HCC in patients with liver cirrhosis caused by the hepatitis C virus. In their study, OPN was more sensitive than AFP for the diagnosis of HCC. On the other hand, HCC risk is known to increase with portal hypertension; therefore, it is still not clear whether OPN in the above-mentioned papers actually reflects the presence of HCC, or if it is just a sign of portal hypertension. This was highlighted in a study by Simao et al. (2015). They examined a total of 90 consecutive patients with alcoholic cirrhosis. OPN levels were significantly increased with advancing Child-Pugh class. They also compared cirrhotic patients with and without HCC and showed that OPN correlated with the stage of HCC, but the same correlation was observed with progressive deterioration of underlying liver function. They concluded that OPN reflects rather the advanced stage of liver cirrhosis than presence of HCC and that it could not be used as a biomarker for HCC.

Zhang et al. (2006) assessed the prognostic value of preoperative plasma OPN levels in 101 patients planned for liver resection due to HCC. Significantly higher plasma OPN levels were found in patients with a recurrence of HCC after resection (214 ng/ml), compared with those without recurrence (153.70 ng/ml). A higher plasma OPN level was an independent prognostic factor for both overall survival and disease-free survival. The authors suggest that the preoperative plasma OPN level can be used as a predictive marker for HCC recurrence and may be helpful in assessing the prognosis of patients with HCC after surgery. The prognostic value of preoperative plasma OPN levels was also studied in patients with early stage HCC (Sun et al. 2010) with similar results. Higher plasma OPN level (cut-off value of 100 ng/ml) was an independent negative prognostic factor for both overall survival and relapse-free survival. Unfortunately, no parameter of portal hypertension or liver dysfunction was compared to OPN regarding the prognostic significance in the abovementioned studies, thus failing to tell us if the OPN provides additional information to the parameters of liver dysfunction or portal hypertension.

Conclusion

OPN is undoubtedly an important cytokine in liver diseases, whose role is not fully understood. Its action is ambivalent, while aggravating liver injury in some situations such as fibrosis or NASH and being protective in other situations such as early alcoholic liver disease or ischemic-reperfusion injury. Suggestions to use anti-OPN treatment in liver disease should be handled with caution and need more studies.

Summary Points

- This chapter focuses on osteopontin (OPN), a multifunctional protein, physiologically expressed in the kidney and bone.
- Under pathological conditions, OPN expression has been found in various organs and attributed to many pathological conditions including inflammation, angiogenesis, fibrosis, and carcinogenesis.
- In the liver, OPN has been shown to contribute to the migration of macrophages into the necrotic areas in liver tissue and to serve as a key cytokine within the extracellular matrix contributing to fibrogenesis.
- OPN thus serves as a cytokine in liver diseases.
- Plasma concentration of OPN is in close relation to portal hypertension in patients with cirrhosis and might be used as a noninvasive parameter of portal hypertension.
- The role of OPN in the liver is nevertheless ambivalent as at some situations, such as early alcoholic hepatitis, it could have a positive role to play.

Acknowledgments Supported by Charles University in Prague (http://www.cuni.cz/UKEN-1.html), No. SVV 260156/2015 and by the Czech Ministry of Health (http://mzcr.cz), No. MZCR-RVO VFN64165.

References

Apte UM, Banerjee A, McRee R, Wellberg E, Ramaiah SK. Role of osteopontin in hepatic neutrophil infiltration during alcoholic steatohepatitis. Toxicol Appl Pharmacol. 2005; 207(1):25–38.

Arriazu, E, Ge X, Leung TM, Magdaleno F, Lopategi A, Lu Y, Kitamura N, Urtasun R, Theise N, Antoine DJ, Nieto N. Signalling via the osteopontin and high mobility group box-1 axis drives the fibrogenic response to liver injury. Gut. 2016 Jan 27, Epub ahead of print.

Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ, Cantor H. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science. 2000;287(5454):860–4.

Bertola A, Deveaux V, Bonnafous S, Rousseau D, Anty R, Wakkach A, Dahman M, Tordjman J, Clement K, McQuaid SE, Frayn KN, Huet PM, Gugenheim J, Lotersztajn S, Le Marchand-Brustel Y, Tran A, Gual P. Elevated expression of osteopontin may be related to adipose tissue macrophage accumulation and liver steatosis in morbid obesity. Diabetes. 2009;58(1):125–33.

Bruha R, Jachymova M, Petrtyl J, Dvorak K, Lenicek M, Urbanek P, Svestka T, Vitek L. Osteopontin: a non-invasive parameter of portal hypertension and prognostic marker of cirrhosis. World J Gastroenterol. 2016;22(12):3441–50.

Coombes JD, Swiderska-Syn M, Dolle L, Reid D, Eksteen B, Claridge L, Briones-Orta MA, Shetty S, Oo YH, Riva A, Chokshi S, Papa S, Mi Z, Kuo PC, Williams R, Canbay A, Adams DH, Diehl AM, van Grunsven LA, Choi SS, Syn WK. Osteopontin neutralisation abrogates the liver progenitor cell response and fibrogenesis in mice. Gut. 2015;64(7):1120–31.

D'Alfonso S, Barizzone N, Giordano M, Chiocchetti A, Magnani C, Castelli L, Indelicato M, Giacopelli F, Marchini M, Scorza R, Danieli MG, Cappelli M, Migliaresi S, Bigliardo B, Sabbadini MG, Baldissera E, Galeazzi M, Sebastiani GD, Minisola G, Ravazzolo R, Dianzani U, Momigliano-Richiardi P. Two single-nucleotide polymorphisms in the 5′ and 3′ ends of the osteopontin gene contribute to susceptibility to systemic lupus erythematosus. Arthritis Rheum. 2005;52(2):539–47.

Da Silva AP, Pollett A, Rittling SR, Denhardt DT, Sodek J, Zohar R. Exacerbated tissue destruction in DSS-induced acute colitis of OPN-null mice is associated with downregulation of TNF-alpha expression and non-programmed cell death. J Cell Physiol. 2006;208(3):629–39.

Fisher LW, Torchia DA, Fohr B, Young MF, Fedarko NS. Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. Biochem Biophys Res Commun. 2001;280(2):460–5.

Franzen A, Heinegard D. Isolation and characterization of two sialoproteins present only in bone calcified matrix. Biochem J. 1985;232(3):715–24.

Ge X, Leung TM, Arriazu E, Lu Y, Urtasun R, Christensen B, Fiel MI, Mochida S, Sorensen ES, Nieto N. Osteopontin binding to lipopolysaccharide lowers tumor necrosis factor-alpha and prevents early alcohol-induced liver injury in mice. Hepatology. 2014;59(4):1600–16.

Gotoh M, Sakamoto M, Kanetaka K, Chuuma M, Hirohashi S. Overexpression of osteopontin in hepatocellular carcinoma. Pathol Int. 2002;52(1):19–24.

Hedtjarn M, Mallard C, Hagberg H. Inflammatory gene profiling in the developing mouse brain after hypoxia-ischemia. J Cereb Blood Flow Metab. 2004;24(12):1333–51.

Huang W, Zhu G, Huang M, Lou G, Liu Y, Wang S. Plasma osteopontin concentration correlates with the severity of hepatic fibrosis and inflammation in HCV-infected subjects. Clin Chim Acta. 2010;411(9–10):675–8.

Husain-Krautter S, Kramer JM, Li W, Guo B, Rothstein TL. The osteopontin transgenic mouse is a new model for Sjogren's syndrome. Clin Immunol. 2015;157(1):30–42.

Hwang SM, Lopez CA, Heck DE, Gardner CR, Laskin DL, Laskin JD, Denhardt DT. Osteopontin inhibits induction of nitric oxide synthase gene expression by inflammatory mediators in mouse kidney epithelial cells. J Biol Chem. 1994;269(1):711–5.

Isoda K, Kamezawa Y, Ayaori M, Kusuhara M, Tada N, Ohsuzu F. Osteopontin transgenic mice fed a high-cholesterol diet develop early fatty-streak lesions. Circulation. 2003;107(5):679–81.

Karamizadeh Z, Kamali Sarvestani E, Saki F, Karamifar H, Amirhakimi GH, Namavar Shooshtarian MH, Ashkani-Esfahani S. Investigation of osteopontin levels and genomic variation of osteopontin and its receptors in type 1 diabetes mellitus. J Endocrinol Invest. 2013; 36(11):1090–3.

Kawashima R, Mochida S, Matsui A, YouLuTu ZY, Ishikawa K, Toshima K, Yamanobe F, Inao M, Ikeda H, Ohno A, Nagoshi S, Uede T, Fujiwara K. Expression of osteopontin in Kupffer cells and hepatic macrophages and Stellate cells in rat liver after carbon tetrachloride intoxication: a possible factor for macrophage migration into hepatic necrotic areas. Biochem Biophys Res Commun. 1999;256(3):527–31.

Kazanecki CC, Uzwiak DJ, Denhardt DT. Control of osteopontin signaling and function by post-translational phosphorylation and protein folding. J Cell Biochem. 2007;102(4):912–24.

Leung TM, Wang X, Kitamura N, Fiel MI, Nieto N. Osteopontin delays resolution of liver fibrosis. Lab Invest. 2013;93(10):1082–9.

Liaw L, Skinner MP, Raines EW, Ross R, Cheresh DA, Schwartz SM, Giachelli CM. The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of

alpha v beta 3 in smooth muscle cell migration to osteopontin in vitro. J Clin Invest. 1995; 95(2):713–24.
Matsue Y, Tsutsumi M, Hayashi N, Saito T, Tsuchishima M, Toshikuni N, Arisawa T, George J. Serum osteopontin predicts degree of hepatic fibrosis and serves as a biomarker in patients with hepatitis C virus infection. PLoS One. 2015;10(3):e0118744.
Mirza M, Shaughnessy E, Hurley JK, Vanpatten KA, Pestano GA, He B, Weber GF. Osteopontin-c is a selective marker of breast cancer. Int J Cancer. 2008;122(4):889–97.
Morales-Ibanez O, Dominguez M, Ki SH, Marcos M, Chaves JF, Nguyen-Khac E, Houchi H, Affo S, Sancho-Bru P, Altamirano J, Michelena J, Garcia-Pagan JC, Abraldes JG, Arroyo V, Caballeria J, Laso FJ, Gao B, Bataller R. Human and experimental evidence supporting a role for osteopontin in alcoholic hepatitis. Hepatology. 2013;58(5):1742–56.
Nabih MI, Aref WM, Fathy MM. Significance of plasma osteopontin in diagnosis of hepatitis C virus-related hepatocellular carcinoma. Arab J Gastroenterol. 2014;15:103.
Nagoshi S. Osteopontin: versatile modulator of liver diseases. Hepatol Res. 2014;44(1):22–30.
Noiri E, Dickman K, Miller F, Romanov G, Romanov VI, Shaw R, Chambers AF, Rittling SR, Denhardt DT, Goligorsky MS. Reduced tolerance to acute renal ischemia in mice with a targeted disruption of the osteopontin gene. Kidney Int. 1999;56(1):74–82.
Oldberg A, Franzen A, Heinegard D. Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. Proc Natl Acad Sci U S A. 1986;83(23):8819–23.
Patouraux S, Bonnafous S, Voican CS, Anty R, Saint-Paul MC, Rosenthal-Allieri MA, Agostini H, Njike M, Barri-Ova N, Naveau S, Le Marchand-Brustel Y, Veillon P, Cales P, Perlemuter G, Tran A, Gual P. The osteopontin level in liver, adipose tissue and serum is correlated with fibrosis in patients with alcoholic liver disease. PLoS One. 2012;7(4):e35612.
Patouraux S, Rousseau D, Rubio A, Bonnafous S, Lavallard VJ, Lauron J, Saint-Paul MC, Bailly-Maitre B, Tran A, Crenesse D, Gual P. Osteopontin deficiency aggravates hepatic injury induced by ischemia-reperfusion in mice. Cell Death Dis. 2014;5:e1208.
Rabenstein M, Hucklenbroich J, Willuweit A, Ladwig A, Fink GR, Schroeter M, Langen KJ, Rueger MA. Osteopontin mediates survival, proliferation and migration of neural stem cells through the chemokine receptor CXCR4. Stem Cell Res Ther. 2015;6:99.
Ramaiah SK, Rittling S. Pathophysiological role of osteopontin in hepatic inflammation, toxicity, and cancer. Toxicol Sci. 2008;103(1):4–13.
Rittling SR, Denhardt DT. Osteopontin function in pathology: lessons from osteopontin-deficient mice. Exp Nephrol. 1999;7(2):103–13.
Rogers SA, Padanilam BJ, Hruska KA, Giachelli CM, Hammerman MR. Metanephric osteopontin regulates nephrogenesis in vitro. Am J Physiol. 1997;272(4 Pt 2):F469–76.
Senger DR, Wirth DF, Hynes RO. Transformed mammalian cells secrete specific proteins and phosphoproteins. Cell. 1979;16(4):885–93.
Shang S, Plymoth A, Ge S, Feng Z, Rosen HR, Sangrajrang S, Hainaut P, Marrero JA, Beretta L. Identification of osteopontin as a novel marker for early hepatocellular carcinoma. Hepatology. 2012;55(2):483–90.
Simao A, Madaleno J, Silva N, Rodrigues F, Caseiro P, Costa JN, Carvalho A. Plasma osteopontin is a biomarker for the severity of alcoholic liver cirrhosis, not for hepatocellular carcinoma screening. BMC Gastroenterol. 2015;15:73.
Sodek J, Ganss B, McKee MD. Osteopontin. Crit Rev Oral Biol Med. 2000;11(3):279–303.
Sorensen ES, Petersen TE. Identification of two phosphorylation motifs in bovine osteopontin. Biochem Biophys Res Commun. 1994;198(1):200–5.
Sorensen ES, Hojrup P, Petersen TE. Posttranslational modifications of bovine osteopontin: identification of twenty-eight phosphorylation and three O-glycosylation sites. Protein Sci. 1995; 4(10):2040–9.
Sun J, Xu HM, Zhou HJ, Dong QZ, Zhao Y, Fu LY, Hei ZY, Ye QH, Ren N, Jia HL, Qin LX. The prognostic significance of preoperative plasma levels of osteopontin in patients with TNM stage-I of hepatocellular carcinoma. J Cancer Res Clin Oncol. 2010;136(1):1–7.

Syn WK, Oo YH, Pereira TA, Karaca GF, Jung Y, Omenetti A, Witek RP, Choi SS, Guy CD, Fearing CM, Teaberry V, Pereira FE, Adams DH, Diehl AM. Accumulation of natural killer T cells in progressive nonalcoholic fatty liver disease. Hepatology. 2010;51(6):1998–2007.

Syn WK, Choi SS, Liaskou E, Karaca GF, Agboola KM, Oo YH, Mi Z, Pereira TA, Zdanowicz M, Malladi P, Chen Y, Moylan C, Jung Y, Bhattacharya SD, Teaberry V, Omenetti A, Abdelmalek MF, Guy CD, Adams DH, Kuo PC, Michelotti GA, Whitington PF, Diehl AM. Osteopontin is induced by hedgehog pathway activation and promotes fibrosis progression in nonalcoholic steatohepatitis. Hepatology. 2011;53(1):106–15.

Syn WK, Agboola KM, Swiderska M, Michelotti GA, Liaskou E, Pang H, Xie G, Philips G, Chan IS, Karaca GF, Pereira Tde A, Chen Y, Mi Z, Kuo PC, Choi SS, Guy CD, Abdelmalek MF, Diehl AM. NKT-associated hedgehog and osteopontin drive fibrogenesis in non-alcoholic fatty liver disease. Gut. 2012;61(9):1323–9.

Terashi T, Aishima S, Taguchi K, Asayama Y, Sugimachi K, Matsuura S, Shimada M, Maehara S, Maehara Y, Tsuneyoshi M. Decreased expression of osteopontin is related to tumor aggressiveness and clinical outcome of intrahepatic cholangiocarcinoma. Liver Int. 2004;24(1):38–45.

Uede T. Osteopontin, intrinsic tissue regulator of intractable inflammatory diseases. Pathol Int. 2011;61(5):265–80.

Urtasun R, Lopategi A, George J, Leung TM, Lu Y, Wang X, Ge X, Fiel MI, Nieto N. Osteopontin, an oxidant stress sensitive cytokine, up-regulates collagen-I via integrin alpha(V)beta(3) engagement and PI3K/pAkt/NFkappaB signaling. Hepatology. 2012;55(2):594–608.

Wai PY, Kuo PC. The role of osteopontin in tumor metastasis. J Surg Res. 2004;121(2):228–41.

Wang Y, Chen B, Shen D, Xue S. Osteopontin protects against cardiac ischemia-reperfusion injury through late preconditioning. Heart Vessels. 2009;24(2):116–23.

Weber GF, Ashkar S, Glimcher MJ, Cantor H. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science. 1996;271(5248):509–12.

Young MF, Kerr JM, Termine JD, Wewer UM, Wang MG, McBride OW, Fisher LW. cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). Genomics. 1990;7(4):491–502.

Zhang H, Ye QH, Ren N, Zhao L, Wang YF, Wu X, Sun HC, Wang L, Zhang BH, Liu YK, Tang ZY, Qin LX. The prognostic significance of preoperative plasma levels of osteopontin in patients with hepatocellular carcinoma. J Cancer Res Clin Oncol. 2006;132(11):709–17.

Zhao L, Li T, Wang Y, Pan Y, Ning H, Hui X, Xie H, Wang J, Han Y, Liu Z, Fan D. Elevated plasma osteopontin level is predictive of cirrhosis in patients with hepatitis B infection. Int J Clin Pract. 2008;62(7):1056–62.

Zohar R, Lee W, Arora P, Cheifetz S, McCulloch C, Sodek J. Single cell analysis of intracellular osteopontin in osteogenic cultures of fetal rat calvarial cells. J Cell Physiol. 1997; 170(1):88–100.

Type VI Collagen: Biological Functions and Its Neo-epitope as Hepatic Fibrosis Biomarker

21

Ki M. Mak and Chien Yi M. Png

Contents

K.M. Mak (✉)
Department of Medical Education/Center for Anatomy and Functional Morphology, Icahn School of Medicine at Mount Sinai, New York, NY, USA
e-mail: ki.mak@mssm.edu

C.Y.M. Png (✉)
Department of Medical Education, Icahn School of Medicine at Mount Sinai, New York, NY, USA
e-mail: max.png@icahn.mssm.edu

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_6

Abstract

Collagen VI forms a filamentous network in connective tissue, linking matrix macromolecules and cells. It is composed of three genetically distinct chains, α1(VI), α2(VI), and α3(VI), with a globular domain at each end. Additionally, three novel chains α4, α5, and α6 have been identified. Intracellularly, collagen VI monomers dimerize and form tetramers, which are secreted and then associate into filaments extracellularly. Gene expression for collagen VI is regulated differently than collagen I or III. Collagen VI interacts with collagen V and fibronectin, contributing to the structural integrity of tissue scaffolds. Moreover, it mediates cell adhesion and promotes migration. Soluble collagen VI acts as a sensor for tissue damage, modulating mesenchymal cell proliferation and survival, matrix homeostasis, and wound healing. Collagen VI-deficient mouse models have been generated, which have been used to investigate collagen VI-related myopathies, mammary carcinogenesis, and skeletal muscle satellite cell homeostasis. Collagen VI expression is upregulated in fibrosis of the adipose tissue and liver. Elevated collagen VI in the circulation is considered an indicator of early alcoholic liver fibrosis. Hepatic stellate cells (HSCs) are likely the source of perisinusoidal collagen VI. Collagen VI immunostaining is enhanced in fibrotic foci, codistributing with collagens I, III, and V. The α2(VI) chain sequesters hepatic matrix metalloproteinase (MMP)-1, MMP-3, and MMP-8 and blocks enzyme activation, preventing fibrolysis. The collagen VI receptor on HSCs offers selective targets for antifibrotic agents. CO6-MMP, a collagen VI neo-epitope generated by the proteolytic actions of MMP-2 and MMP-9, serves as a noninvasive biomarker in experimental liver fibrogenesis.

Keywords

Type VI collagen • Filamentous collagen • Soluble collagen VI • Collagen VI assembly • Matrix metalloproteinase • Hepatic stellate cells • Immunohistochemistry • Liver fibrosis biomarker • Collagen VI neo-epitope • Protein fingerprint

List of Abbreviations

BM	Bethlem myopathy
CCl_4	Carbon tetrachloride
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
HSA	Human serum albumin
HSC	Hepatic stellate cell
MMP	Matrix metalloproteinase
MMTV-PyMT	Mammary tumor virus-polyoma middle T antigen
TGF-β	Transforming growth factor-β

Key Facts of Liver Fibrosis

- Liver fibrosis is a chronic liver disease that results from wound healing in response to liver injury caused mainly by viral infections (hepatitis B and C), alcohol abuse, cholestasis, steatosis, toxins, and drugs. Cirrhosis is the end stage of liver fibrosis and is also a risk factor for developing hepatocellular carcinoma.
- Liver cirrhosis is a global health problem causing over one million deaths annually. In the USA, the prevalence of cirrhosis is estimated at 270 per 100,000 of population or approximately 675,000 patients. Cirrhosis is the 11th leading cause of death in the USA.
- Orthotopic liver transplantation is the only therapy that improves the survival rate in patients with cirrhosis, but the availability of liver transplants is limited by the shortage of donor livers. In the USA, there are 17,000 adults and children awaiting liver transplantation, and every year more than 1,500 people die waiting for a donor liver to be available. As an alternative, transplantation of fetal human liver stem cells or adult human liver stem/progenitor cells is being explored.
- Nevertheless, fibrosis and even cirrhosis can be reversed by the elimination of the underlying cause of liver disease. Thus, eradication of HBV or HCV has been shown to improve cirrhosis. Studies of animal models in reversibility have provided clues to underlying mechanisms.
- Liver biopsy is the gold standard for the clinical assessment of liver fibrosis; however, the biopsy is invasive with possible risks of injury to the patient. Moreover, the histological diagnosis is hampered by biopsy sample size and variations and a lack of consensus between pathologists.
- Currently, a single noninvasive marker for assessing the amount of fibrosis in a patient is unavailable. Very few molecules have been satisfactorily validated as fibrosis biomarkers. Combining measurement of several markers could help discriminate minimal fibrosis from advanced fibrosis, but this approach cannot entirely replace liver biopsy.

Definition of Words and Terms

Collagen	A macromolecule composed of three polypeptides arranged in helical chains. The collagen polypeptides contain two unique amino acids, hydroxyproline and hydroxylysine. To date, there are 28 types of collagen. Morphologically, types I, II, and III collagens occur as banded fibrils with the characteristic 64-nm periodicity and are called fibrillar collagens, while other collagens lack banding and are called non-fibrillar collagens. Collagen fibers are found in the connective tissue, providing support and tensile strength to the tissue.
Extracellular matrix (ECM)	Connective tissue consists of cells and tissue; the latter is known as ECM that surrounds the cells. It is composed of

	ground substances, fibers, and structural proteins. It is the medium through which nutrients and metabolites are transported between the blood circulation and organs.
Filaments	Threadlike protein molecules, ranging from 5–6 to 16 nm in diameter. The three types of filaments are actin, a thin filament; cytokeratin, an intermediate filament; and myosin, a thick filament. Filaments are found within the cells mainly for providing structural support and contraction.
Filamentous collagen	A type of collagen that exhibits the diameters of filaments and lacks the banding pattern as seen in fibrillar collagens. Type VI collagen is a filamentous collagen.
Immunohistochemistry	An immunological-based histochemical technique for detecting antigens (proteins) in cells or tissues using antibodies binding specifically to the antigens. Visualization of the antibody and antigen reaction in the tissue with the microscope (fluorescent or light) is achieved by labeling the antibody with a fluorescent molecule or an enzyme. Immunohistochemistry is widely used for diagnostic purposes as well as for research.
Liver fibrosis	Chronic liver disease characterized by excess deposition of ECM proteins particularly collagens in the extracellular space of the liver, namely, space of Disse, stroma of portal tracts, and wall of central veins.
Liver cirrhosis	End stage of liver fibrosis characterized by extensive scarring of the liver parenchyma causing obstruction to blood flow with complications including portal hypertension, jaundice, esophageal varices, ascites, caput medusae, and encephalopathy. The histological hallmark of cirrhosis is the formation of fibrotic nodules.
Liver fibrosis biomarkers	Molecules used for monitoring the development of fibrosis. These are related to liver function, extracellular matrix synthesis and degradation, or fibrogenic-related cytokines.
Matrix metalloproteinase (MMP)	Endopeptidase (proteolytic enzyme) capable of degrading extracellular matrix macromolecules in particular collagens and some other matrix proteins.
Neo-epitopes	Cleavage peptide fragments generated by the proteolytic action of an enzyme such as matrix metalloproteinase (MMP). The fragments are used as immunogen to generate antibodies that specifically react with the epitopes, which can be measured by enzyme-linked immunosorbent assay. Neo-epitopes are used in the development of noninvasive biochemical biomarkers for monitoring fibrosis development.

Introduction

The filamentous type VI collagen is present in most connective tissue matrices where it forms a flexible filamentous network, linking matrix macromolecules and cells. This chapter begins with an introduction to the nomenclature of type VI collagen and an overview of its molecular structure. The biosynthesis, assembly, degradation, and biological functions of collagen VI, as well as mouse models of collagen VI deficiency, are highlighted. This chapter also covers the role of soluble collagen VI as a stimulator of cell growth, promoter of cell survival, sensor molecule for tissue damage, modulator of connective tissue matrix homeostasis, mediator of mouse mammary tumorigenesis, and regulator of the self-renewal capacity of skeletal muscle satellite cells and muscle regeneration. In addition, the contribution of collagen VI to adipose tissue fibrosis is discussed. Finally, this chapter specifically reviews the involvement of collagen VI in liver fibrogenesis, the value of collagen VI as an indicator of early liver fibrosis, and the technological development of collagen VI neo-epitope as a noninvasive biomarker of liver fibrosis.

Nomenclature and Structure

Type VI collagen – designated by Furthmayr et al. (1983) – is classified as a non-fibrillar collagen, as opposed to interstitial fibrillar collagens I, II, and III. Along with type IV collagen of the basement membrane, collagen VI is grouped under the network-forming collagens (Knupp and Squire 2005). It is widely distributed in most connective tissue matrices (von der Mark et al. 1984; Keene et al. 1988; Marcelino and McDevitt 1995; Kuo et al. 1997). Chemically, the collagen VI molecule is a heterotrimeric collagenous glycoprotein made of three genetically distinct α-chains, α1(VI), α2(VI), and α3(VI). These chains differ in molecular mass: 140 kDa for α1 and α2 chains and 250 kDa for α3 chain (Weil et al. 1988). The monomer consists of two globular domains at the N- and C-terminals that are connected by a 105-nm-long triple helix (Chu et al. 1988; Keene et al. 1988; Baldock et al. 2003; Knupp and Squire et al 2005). Uniquely, the triple helical domains are extensively linked by intra- and interchain disulfide bonds that likely endow the collagen VI molecules with a high thermal stability and protease resistance. The cDNAs of the three constituent chains of human collagen VI have been cloned and a large portion of the amino acids has been sequenced (Chu et al. 1988). Of note, there are several Gly-Y-X triplet interruptions of the amino acid sequence that are thought to provide flexibility to the collagen VI molecules. This feature differs from the non-interrupted Gly-Y-X repeats in the fibrillar collagens that confer rigidity to the molecules and mechanical strength to the fibers. Another unique structural feature of collagen VI is that it contains the sequence Arg-Gly-Asp (RGD)-dependent cell attachment sites that are likely involved in interaction with specific cell receptors of the integrin family proteins. The genes for α1(VI) and α2(VI) chains are located on chromosome 21, while the α3(VI) gene is located on chromosome 2

(Weil et al. 1988). The major mRNA species encoding the chains of collagen VI have sizes of 4.2 kb (α1), 3.5 kb (α2), and 8.5 kb (α3).

More recently, three novel collagen VI genes (*COL6A4*, *COL6A5*, *and COL6A6* located at a single locus on human chromosome 3q22.1) that encode the α4(VI), α5 (VI), and α6(VI) chains have been identified (Gara et al. 2008; Fitzgerald et al. 2008). These chains may substitute for the α3 chains, probably forming α1α2α4, α1α2α5, and α1α2α6 heterotrimers. Unlike the α1(V), α2(V), and α3(V) subunits, these collagen VI chains display a highly restricted tissue distribution pattern (Gara et al. 2011; Sabatelli et al. 2011), raising the possibility of the tissue-specific role for the chains in collagen VI assembly and function.

Synthesis, Assembly, and Secretion

The biosynthesis of type VI collagen was studied in cultured human fibroblasts (Engvall et al. 1986) and chick embryo fibroblasts (Colombatti et al. 1987) using [^{35}S]methionine metabolic labeling of cells. Two labeled polypeptides of 140 and 260 kDa were identified in the cell layer lysates, matrices, and media of the human fibroblast culture, while three polypeptides of 150 kDa, 140 kDa, and 260 kDa were identified in the chick embryo fibroblast culture media. These give rise, after pepsin digestion, to α1(VI), α2(VI), and α3(VI), respectively. Pulse-chase experiments in the embryo chick cells indicated that more than 60% of the labeled type VI collagen was present in the culture medium after a 4-h chase duration. In both cell systems, the amounts of polypeptides deposited extracellularly were dependent on the presence of ascorbic acid and hydroxylation of prolines and lysines in the collagenous domains, as observed in fibrillar collagens (Engvall et al. 1986; Colombatti and Bonaldo 1987). But, unlike the fibrillar collagens, no proteolytic processing of the N- and C-terminal domains of the polypeptide chains occurred in collagen VI biosynthesis. Another study has shown that recombinant chicken α1(VI), α2(VI), and α3(VI) collagen chains can form monomers, dimers, and tetramers in NIH/3T3 cell lines. These molecules were secreted into the culture matrix, forming fibrillar meshwork (Colombatti et al. 1995). This model may offer a tool for the analysis of type VI collagen assembly and deposition.

The collagen VI polypeptide structures from the human fibroblast culture have been examined by electron microscopy after rotary shadowing (Engvall et al. 1986). The images in Fig. 1 revealed that the cell layer extracts contain monomers, dimers, and tetramers of collagen VI, the culture matrices contain both tetramers and oligomers, and the culture media contain predominantly tetramers. The distribution of these molecules in various compartments of the culture likely reflects the various stages of collagen VI assembly in vivo as described below. Based on the data of rotary shadowing electron microscopy, physical and biochemical analyses, the sequence of events of collagen VI's intracellular assembly has been established (Furthmayr et al. 1983; Engel et al. 1985; Engvall et al. 1986; Colombatti et al. 1987; Baldock et al. 2003; Knupp and Squire 2005). In this model, as illustrated in Fig. 2 (Mak et al. 2014),

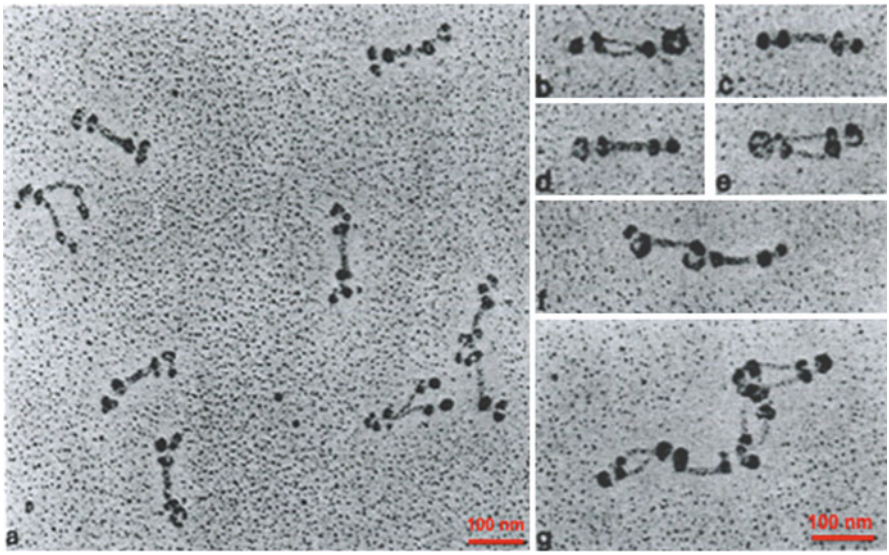

Fig. 1 Rotary-shadowed preparation of type Vl collagen. Electron microscopic images of type VI collagen after rotary shadowing. Collagen VI was isolated from the medium and or extracellular matrix extracts of cultured human lung fibroblasts. (**a**) shows a field of representative molecules in the medium. Most of the structures are ~150 nm long and composed of a central thin rod, 60 nm long, and two flanking globules. Extending from each of these globules is a thin strand with another globule at its outer end. These structures likely represent the tetramers secreted from the cells. (**b**–**g**) show selected collagen VI molecules isolated from the culture matrix extracts. They appear to be tetramers or oligomers of collagen VI. The structure in (**g**) probably represents four tetramers with end-to-end association forming a short beaded filament ()

two triple helical monomers of 105 nm in length form a dimer in an antiparallel manner with a 75-nm overlap. Two dimers associate into a tetramer, with the chains stabilized by disulfide bonds (Furthmayr et al. 1983; Chu et al. 1988; Weil et al. 1988). Following secretion into the extracellular matrix (ECM), the tetramers assemble into filaments by end-to-end accretion, forming thin fibrils with prominent knobs at a periodicity of about 110 nm—so-called beaded filaments (Bruns et al. 1986; Engvall et al. 1986; Keene et al. 1988). The fibrils display a width of 6–10 nm; hence, collagen VI is described as filamentous (Amenta et al. 1988) or microfibrillar (Baldock et al. 2003).

Gene Expression

Collagen VI is abundantly expressed by cultured fibroblasts. Expression of collagen VI mRNA and its protein production were assessed in the human skin fibroblast culture, and the changes were compared with those of collagens I and III and fibronectin, which are known to be regulated in a coordinated fashion (Hatamochi

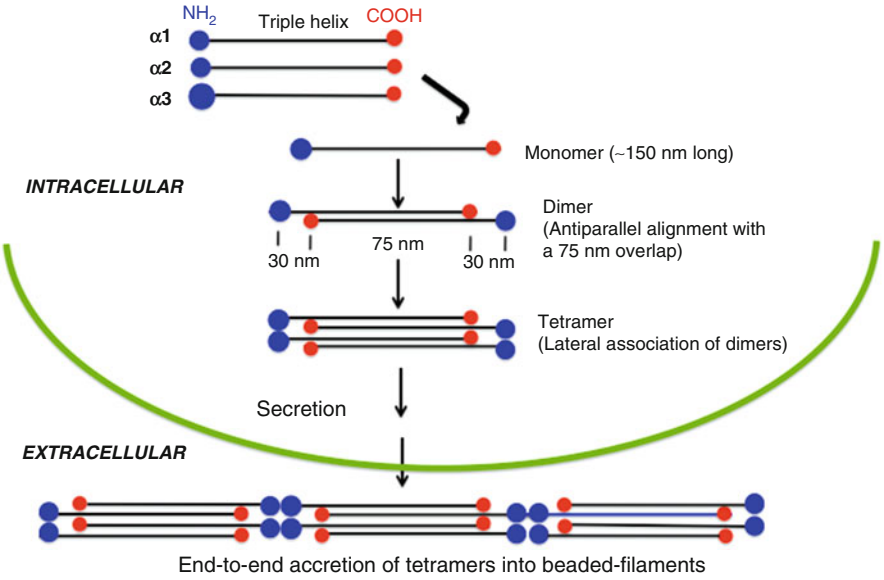

Fig. 2 Type Vl collagen assembly. This schema illustrates collagen VI formation in the cell and its secretion into the extracellular space. The assembly of monomers into dimers and then into tetramers occurs intracellularly. Following secretion, the tetramers associate end-to-end into beaded microfilaments. Note that the C-terminal globular domains contact the adjacent helices in the inner regions of the dimers that are stabilized by disulfide bonds (From Mak et al. 2014, Austin Biomarkers and Diagnosis 1(2): id1012, 2014)

et al. 1989). When the fibroblasts were grown at high densities or in a contracting collagen gel (conditions that reduce the proliferative capacity of the cells), a two- to threefold upregulation of the α1(VI), α2(VI), and α3(VI) mRNA chains was observed along with an increase in the corresponding proteins. Concurrently, there were only minimal changes for the mRNA levels of collagens I and III and fibronectin. Transformation of mouse 3T3 fibroblasts with tumor-promoting phorbol esters did not change the collagen VI mRNA level, but it did cause a three- to fivefold reduction in the mRNA levels of other matrix proteins. These data indicate that expression of α1(VI), α2(VI), and α3(VI) subunits is differently regulated in cultured fibroblasts than interstitial fibrillar collagens I and III and fibronectin. Moreover, in response to the pro-fibrotic mediator transforming growth factor-β (TGF-β), human skin fibroblasts selectively expressed the α3(VI) subunit mRNA (227% of control), while the levels of α1(VI) and α2(VI) chains were not changed (Heckmann et al. 1992). Additionally, collagen VI protein was increased in the culture medium and cell layer extracts (170% of control). Therefore, the regulation of α3(VI) gene expression by TGF-β is critical for the control of collagen VI synthesis and may affect the deposition of the collagen VI molecule in the ECM. These notions are compatible with a study that used recombinant type VI collagen (Colombatti et al. 1995).

Enzymatic Degradation

Collagen VI is made and deposited in the ECM where it becomes associated with fibrillar collagens I, III, and V, and together these collagens contribute to the structural integrity of the tissue scaffold of the ECM. However, the ECM is constantly remodeled even in normal conditions with a balanced production and degradation of the ECM proteins so as to achieve a balanced state of homeostasis for proper functioning of the tissue. An imbalanced remodeling of the ECM will cause pathological changes. Consequently, like other collagen types, collagen VI is subject to enzymatic digestion, contributing to ECM remodeling in normal and pathological conditions. Several matrix-degrading enzymes, namely, serine proteinases, matrix metalloproteinases (MMPs), and lysosomal enzymes, which act on collagen VI, are described below.

Intact collagen VI filaments are susceptible to degradation by serine proteinases, which are enzymes typically secreted by neutrophils and mast cells, but are resistant to both degradation by MMP-1, MMP-2, MMP-3, and MMP-9 that commonly degrade other collagens, and to bacterial collagenase (Kielty et al. 1993). These properties of collagen VI suggest that it is a relatively stable molecule of the ECM, consistent with its role in matrix organization. The susceptibility of collagen VI to digestion by serine proteases suggests that collagen VI may be targeted for degradation primarily during physiological tissue turnover with inflammatory cell involvement and in early inflammatory lesions. However, Myint et al. (1996) demonstrated that activated MMP-2 cleaves collagen VI extracts from normal human cornea into lower molecular weight fragments. Additionally, recent data indicate that type VI collagen can be cleaved by MMP-2 and MMP-9 in vitro with the generation of neo-epitope peptide fragments that can be used as markers for assessing collagen VI turnover during hepatic fibrogenesis (Veidal et al. 2011). Furthermore, degradation of collagen VI has been reported for fibroblasts of periosteal explants via phagocytosis and subsequent digestion by lysosomal enzymes (Everts et al. 1995).

Biological Functions

Interaction with ECM Components

In most connective tissue matrices, collagen VI forms a flexible, branching filamentous network that surrounds the fibers of the major fibrillar collagens I, II and III, and V; hence, collagen VI is sometimes called fibril-associated collagen. It anchors nerves, blood vessels, and mesenchymal cells into place, partly through interconnections with collagen IV in endothelial cell basement membranes (Amenta et al. 1988; Kenne et al.1988; Kuo et al. 1997). More notably, it connects the fibrils of fibronectin in the ECM and binds matrix components, including hyaluronan, decorin, syndecan, von Willebrand factor, MMPs, and growth factors (Mak et al. 2014). Hence, collagen VI has been called a connecting protein, linking components of the ECM (Amenta et al. 1988; Keene et al. 1988), as schematized

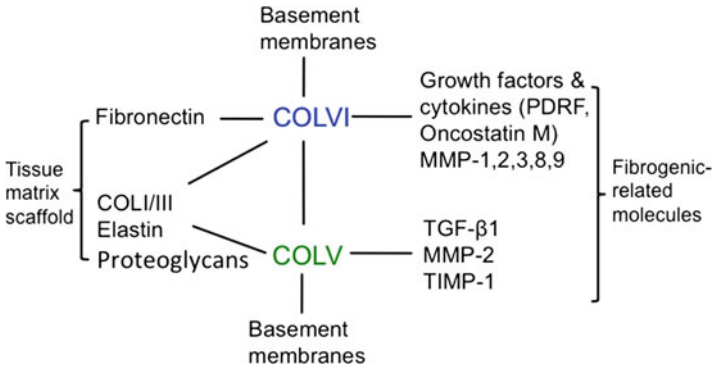

Fig. 3 Overview of type Vl collagen and matrix molecule interactions. Collagen VI (COLVI) interacts with collagen V (COLV), and in turn, both collagens are linked to matrix fibrillar collagens (COLI and III) and structural proteins, contributing to tissue matrix scaffold. Collagen VI as well as collagen V serves as reservoir for fibrogenic-related molecules, thus regulating their availability for fibrogenesis and fibrolysis. Both collagens VI and V have contacts with basement membranes, but are not integral basement membrane components. Collagen VI, but not collagen V, shows structural connection with fibronectin, a prominent matrix macromolecule. *PDGF* platelet-derived growth factor, *TIMP-1* tissue inhibitor of metalloproteinase-1

in Fig. 3. Strikingly, in the skin dermis, collagen VI filaments (marked by immunogold particles) could be seen interweaving with collagen V fibrils that in turn link other fibrillar collagens, elastic fibers, and proteoglycans, forming a network resembling wickerwork (Kobayasi and Karismark 2006). It is conceivable that these ECM structural proteins serve to provide support for the collagenous scaffold of the dermal tissue matrix.

Interaction with Fibronectin

Fibronectin is a multifunctional matrix glycoprotein with multiple domains that plays an important role in the interaction between cells and the surrounding ECM (Schuppan 1990). Electron microscopic study of replicas of whole-mounted cultured cells and matrix revealed that the filaments of collagen VI interconnect with the fibrils of fibronectin at some discrete sites (Sabatelli et al. 2001). The interaction is thought to render the three-dimensional configuration of the fibronectin fibrils. This notion is corroborated by a study that used cultured fibroblasts obtained from *Col6a1* null mutant mice that lack the assembly of collagen VI and the capacity to secrete collagen VI into the ECM (Bonaldo et al. 1998). Consequently, the absence of collagen VI in the matrix of cultured fibroblasts resulted in a loss of the three-dimensional organization of the fibronectin fibrils. This effect could compromise various cellular functions. Additionally, patients affected by Bethlem myopathy (BM) present with an

abnormal organization of fibronectin, possibly due to a drastically reduced secretion of collagen VI by fibroblasts. Thus, immunofluorescent labeling of collagen VI in skin fibroblast cultures derived from BM patients has been considered a useful addition to current diagnostic services for BM (Hicks et al. 2008).

Stimulation of Cell Growth

Soluble collagen VI, which is the pepsin-solubilized triple-helical core fragment of the native collagen VI, is released from the filamentous collagen VI in response to tissue damage and inflammation (Atkinson et al. 1996; Rühl et al. 1999a, b; Schuppan et al. 2001). In contrast to other collagens, soluble collagen VI stimulates proliferation of normal 3T3 fibroblasts and transformed fibrosarcoma cells in culture in the absence of growth factors (Atkinson et al. 1996). The cell growth effect of collagen VI is mediated by signal transduction cascades that involve induction of tyrosine phosphorylation of proteins, including paxillin, focal adhesion kinase, and the mitogen-activated protein kinase erk2 (Rühl et al. 1999a). Furthermore, these signaling cascades appear to be independent of the integrin receptor protein α2ß, which mediates cell adhesion. The signaling transduction appears to require an aggregation of the collagen VI receptors or occupancy of the receptors by the native helical structure of collagen VI; interestingly, the effects on cell growth can be inhibited by single chains of collagen VI—prepared from the native collagen VI by reduction and alkylation with methylene imine (Rühl et al. 1999a; Schuppan et al. 2001).

Promotion of Cell Survival

Soluble collagen VI promotes survival of fibroblasts cultured in a serum-free medium through an anti-apoptotic mechanism involving downregulation of the proapoptotic Bax and upregulation of cyclins A, B, and D1 (Rühl et al. 1999b), whereas collagen I tested under the same experimental conditions had no anti-apoptotic action. The pro-survival action of collagen VI has also been seen in hepatic stellate cells (HSCs), the principal fibrogenic cells of the liver (Ruehl et al. 1999). These events are mediated, in part, by the activity of the transmembrane receptor NG2/chondroitin sulfate proteoglycan (Schuppan et al. 2001), which binds collagen VI (Tillet et al. 1997). This cellular interaction was examined by electron microscopy after rotary shadowing of a mixture of native NG2 and collagen VI, which revealed an alignment of collagen VI tetramers with the central region between the two N- and C-terminal globular regions of NG2. Furthermore, binding of collagen VI to the integrins α1β1and α2β1 facilitates cell adhesion, spreading, and migration of smooth muscle cells and corneal fibroblasts, as well as invasion of various tumor cell lines in primary culture (Howell and Doane 1998).

Sensor for Tissue Damage and Modulator of Matrix Homeostasis

While the filamentous collagen VI is important in maintaining the integrity of ECM, the soluble form of collagen VI has been proposed as a sensor molecule for tissue damage, stimulating surrounding mesenchymal cell growth and promoting cell survival and wound healing. Collagen VI, along with collagens I, III, IV, and V, serves as a reservoir for cell receptors, platelet-derived growth factor, oncostatin M, and MMP-1, MMP-3, MMP-8, MMP-2, and MMP-9 (Somasundaram and Schuppan 1996; Somasundaram et al. 2002; Freise et al. 2009) and therefore regulates their availability and activity in normal tissue turnover, in wound healing, and in the disease. In response to needs, growth factors are released and act on nearby fibrogenic cells in the matrix, initiating cell proliferation and mediating fibrogenic activity, while MMPs act on their collagen and protein substrates, facilitating tissue turnover. For these reasons, type VI collagen is regarded as a key modulator of matrix homeostasis.

Animal Models of Collagen VI Deficiency

Murine models of collagen VI deficiency have been described, namely, *Col6a1* (Donaldo et al. 1998), *Col6a3* (Pan et al. 2013), *and* $Col6a3^{+/d16}$ (heterozygous exon 16 deletion) (Pan et al. 2014). These animal models have been employed to investigate the molecular pathogenesis of collagen VI-related congenital Bethlem and Ullrich myopathies and skeletal muscle satellite cell homeostasis. Additionally, the collagen VI knockout ($Col6a1^{-/-}$) mice—in the background of the mammary tumor virus-polyoma middle T antigen (MMTV-PyMT) (Guy et al. 1992; Iyengar et al. 2005; Park and Scherer 2012)—have been used as a mammary cancer model.

Specialized Roles

Mammary Gland Tumorigenesis

Expression of collagen VI is upregulated during the progression of murine mammary tumors. Studies using $Col6a1^{-/-}$ mice (MMTV-PyMT mice) (Guy et al. 1992; Iyengar et al. 2005) have provided evidence indicating that adipocyte-derived soluble collagen VI exerts a stimulatory effect on the hyperplasia of mammary ductal epithelial cells, leading to primary tumor growth at the early stage of mammary tumorigenesis (Iyengar et al. 2005). Additionally, the lack of collagen VI in the knockout mice promotes apoptotic cell death of mammary epithelial cells, thereby reducing the likelihood of tumor expansion (Khan et al. 2009). Moreover, the carboxy-terminal fragment of collagen α3 (VI) chain, a proteolytic product of the full-length molecule, was found to exert pro-mitogenic and pro-survival actions in part by signaling through the collagen

VI binding proteoglycan NG2 receptor on the surface of malignant ductal epithelial cells (Iyengar et al. 2005; Khan et al. 2009). The action leads to the activation of Akt and β-catenin signaling pathways, resulting in the mitogenic response. Therefore, collagen VI secreted by adipocytes, acting as a paracrine factor, appears to mediate a critical interaction between adipocytes and tumor cells in the tumor-stroma microenvironment. In line with these data, Park and Scherer (2012) showed that endotrophin, the C-terminal cleavage product of the α3(VI) chain derived from adipose tissue, serves as a major mediator of collagen VI-stimulated mammary tumorigenesis by augmenting both mammary tumor growth and metastasis in PyMT/endotrophin mice. These effects are associated with the induction of adipose tissue fibrosis, angiogenesis, inflammation, and epithelial-mesenchymal transition of tumor cells, which are mediated in part through the upregulated signaling pathway of TGF-β, a pro-fibrotic factor.

Skeletal Muscle Regeneration

As an ECM protein of the skeletal muscle, collagen VI is a critical component of the satellite cell niche (Urciuolo et al. 2013). Deficiency of collagen VI in the skeletal muscle of mice is associated with muscular disorder resembling BM (Bonaldo et al. 1998). Investigation in collagen VI*a*1 null mice has shown that the lack of collagen VI causes impaired muscle regeneration accompanied by reduced capability of satellite cell to undergo self-renewal after injury to the skeletal muscle. When collagen VI is reinstated in vivo by grafting wild-type fibroblasts, the muscle stiffness associated with *Col6a1*$^{-/-}$ mice is ameliorated, and the satellite cell defects in self-renewal are corrected. Thus, it was proposed that collagen VI plays a regulatory role for satellite cell homeostasis.

Adipose Tissue Fibrosis

Collagen VI is abundantly produced and secreted by adipocytes (Iyengar et al. 2005; Scherer et al. 1998; Pasarica et al. 2009). In fact, adipose tissue is the single most abundant source of collagen VI systemically (Scherer et al. 1998). In adipose tissue, collagen VI forms an integral component of the extracellular scaffold for adipocytes and has a fibrogenic role in the development of obesity. Fibrosis of adipose tissue increases adipose tissue rigidity and constraints adipocyte expansion, thereby contributing to metabolic dysfunction. Conversely, the absence of collagen VI associated with collagen VI knockout in ob/ob mice appears to cause an unlimited expansion of individual adipocytes, resulting in metabolic benefits with a substantial improvement of body energy homeostasis (Khan et al. 2009). Clinically, obese human adipose tissue exhibits large areas of fibrosis containing increased deposition of collagen VI coincident with enhanced gene expression of α3(VI) (Pasarica et al. 2009) or α1(VI) (Spencer et al. 2010). Additionally, the fibrotic change

correlates with increased inflammatory infiltrate of activated pro-fibrotic macrophages, body mass index (Pasarica et al. 2009), and insulin resistance (Spencer et al. 2010). However, in another study of human subjects, McCulloch et al. (2015) found that α3(VI) gene expression is not increased in obesity and does not correlate with impaired metabolic parameters or respond to variations in insulin. Also, these findings are at variance with the previous observations in obese, diabetic (ob/ob) mice (Khan et al. 2009). Instead, it was found that leptin, a pro-fibrotic and pro-inflammatory cytokine, downregulates *COL6A3* expression, suggesting that the cytokine can regulate ECM composition of adipose tissue.

In addition to displaying tumor-promoting effects (Park and Scherer 2012) as discussed above, endotrophin stimulates adipose tissue fibrosis. In endotrophin-overexpressing transgenic mice that were fed a high-fat diet, endotrophin was found to exert a local effect on the histogenesis of fibrosis in adipose tissue, leading to a systemic elevation of pro-inflammatory cytokines and insulin resistance in many other tissues (Sun et al. 2014). Blocking endotrophin with a neutralizing antibody reduces these adverse effects, emphasizing that endotrophin is a potential therapeutic target.

Hepatic Fibrosis

Immunohistochemistry

Immunohistological studies of the human liver revealed that type VI collagen is present in the liver lobules, stroma of portal tracts, wall of intralobular veins and Glisson's capsule (Griffiths et al. 1992; Loreal et al. 1992). Within the lobules, collagen VI immunostaining showed two distribution patterns: either uniform in the perisinusoidal space of Disse (Loreal et al. 1992) or stronger in the centrilobular and mid-lobular areas compared to the periportal area (Griffiths et al. 1992). By light immunohistochemistry, collagen VI immunoreactivity was detected in perisinusoidal HSCs (Griffiths et al. 1992), and by immunoelectron microscopy, the immunostain was localized to the HSC endoplasmic reticulum (Loreal et al. 1992), thereby disclosing the cellular source of collagen VI. Figure 4 illustrates positive collagen VI staining of human HSCs (Mak et al. 2014). In the space of Disse, amorphous or microfibrillar materials containing collagen VI immunostain were observed around and between banded fibrils, suggesting that this collagen interconnects collagens I and/or III fibers (Griffiths et al. 1992; Loreal et al. 1992). It might be presumed that collagen VI determines the organization of the fibrillar collagens in fibrogenesis of space of Disse.

In alcoholic fibrosis and cirrhosis, intense collagen VI staining was present in the developing fibrous septa and bridging septa of cirrhotic nodules (Loreal et al. 1992). In biliary cirrhosis, strong staining for collagen VI was noted around proliferating bile ductules within the developing fibrous septa or the established septa of the cirrhotic liver (Griffiths et al. 1992). Little data, however, is available on the

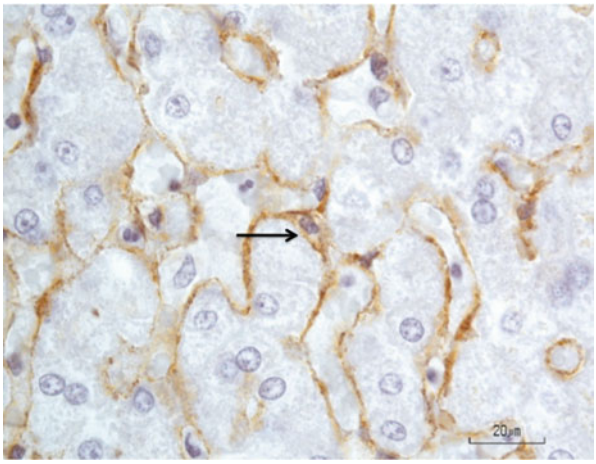

Fig. 4 Human hepatic stellate cells. Immunoperoxidase staining of human hepatic stellate cells for collagen VI. Liver tissue was fixed in formalin and embedded in paraffin. For immunohistochemistry, formalin-fixed and deparaffinized liver section was treated sequentially with a rabbit polyclonal collagen VI antibody (Novus Biologicals, Littleton, CO), anti-rabbit polymer-horseradish peroxidase (Dako Carpinteria, CA), and the chromogen diaminobenzidine tetrahydrochloride to yield a brown reaction product, with buffer washes between steps. Nuclei were counterstained with hematoxylin. The *arrow* points to a positively stained perisinusoidal stellate cell. The immune deposits (*brown*) of collagen VI could be seen in the cell body and its cell process along the sinusoidal border. The unstained clear space in the cytoplasm represents lipid droplets, characteristic of hepatic stellate cells (From Mak et al. 2014, Austin Biomarkers and Diagnosis 1(2): id1012, 2014)

distribution of collagen VI in progressive stages of liver fibrosis and its codistribution with fibrillar collagens I, III, and V in the human liver. Fibrosis of the liver is prevalent in elderly cadavers, even when liver disease is not indicated as the cause of death (Mak et al. 2012). In cadaveric livers with progressive stages of fibrosis, there is an enhanced immunostaining for collagen VI in the parenchyma showing severe perisinusoidal/pericellular fibrosis, which appears to co-distribute with the increased staining for fibrillar collagens I, III, and V in the fibrotic foci (Figs. 5, 6, and 7). In the fibrous septa of septal fibrosis, bridging fibrosis, and cirrhosis, the fibrous matrices show strong immunostaining for collagen VI along with collagens I, III, and V (Fig. 8). These immunohistological data point to a role for collagen VI in the integration of the fibrillar collagens in the histogenesis of fibrotic lesions, thereby contributing to the progression of hepatic fibrosis.

In experimental fibrosis, gene expression of collagen VI was examined by in situ hybridization in conjunction with immunohistochemical detection of the protein in the liver of rats after acute CCl_4 injury (Takahara et al. 1995). The α2(VI) collagen mRNA levels were elevated three days after the CCl_4 treatment accompanied by upregulation of the mRNA for collagen I. The mRNA signals for collagens VI and I

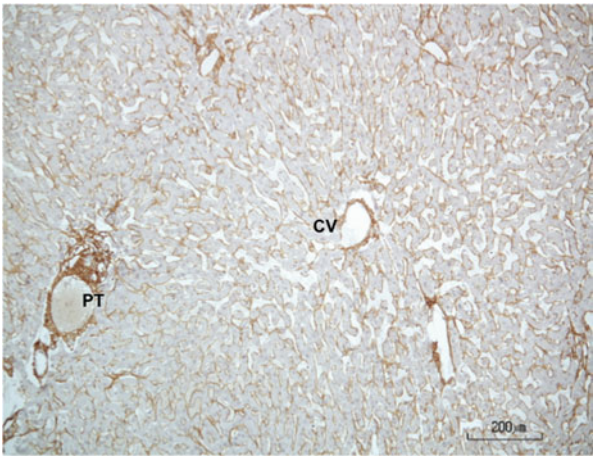

Fig. 5 Lobular distribution of type Vl collagen in the liver. Immunoperoxidase staining showing distribution of collagen VI in the liver lobule of human cadaver. The tissue from embalmed elderly cadavers was processed for paraffin embedment as previously described (Mak et al. 2012), and immunoperoxidase staining was performed as indicated in Fig. 4. This liver with a nearly normal histology shows a uniform distribution of collagen VI immunostain (*brown*) along the sinusoidal lining of the liver lobule. Immunostaining is also seen in the stroma of portal tracts and rims of central veins. Hematoxylin counterstained. *PT* portal tract, *CV* central vein

were concentrated around the perivenous area with a corresponding increased staining for the protein of collagen VI. With longer duration of treatment of 14 weeks, collagen VI mRNA levels did not change, while collagen VI protein was detected in the developing fibrous septa. It was concluded that the collagen VI gene is activated early in the fibrotic process, resulting in production of collagen VI protein. Along this line, others have described an increased deposition of collagen VI, along with collagens I, III, and V, in the developing fibrous septa and fibrotic bands of cirrhotic livers of rats caused by CCl_4 (Martinez-Hernandez and Amenta 1993).

Interactions with MMPs

MMPs are critical modulators of hepatic fibrogenesis (Arthur 2000). Their functions are to degrade interstitial fibrillar native collagens (MMP-1, MMP-8, and MMP-13), basement membrane type IV collagen, denatured fibrillar collagens (gelatinases MMP-2 and MMP-9), non-collagenous matrix proteins, and proteoglycans (stromelysin-1/MMP-3). Apart from being substrates for MMPs, collagens themselves also sequester and modulate the availability of MMPs, particularly of the catalytic inactive proforms. As shown by immunohistochemistry, the alcoholic cirrhotic liver displays an enhanced immunostaining for collagen VI in the matrix of the fibrous septa, which appears to co-distribute with the immunoreactivity of MMP-1 and MMP-3, suggesting MMP binding to collagen VI (Freise et al. 2009).

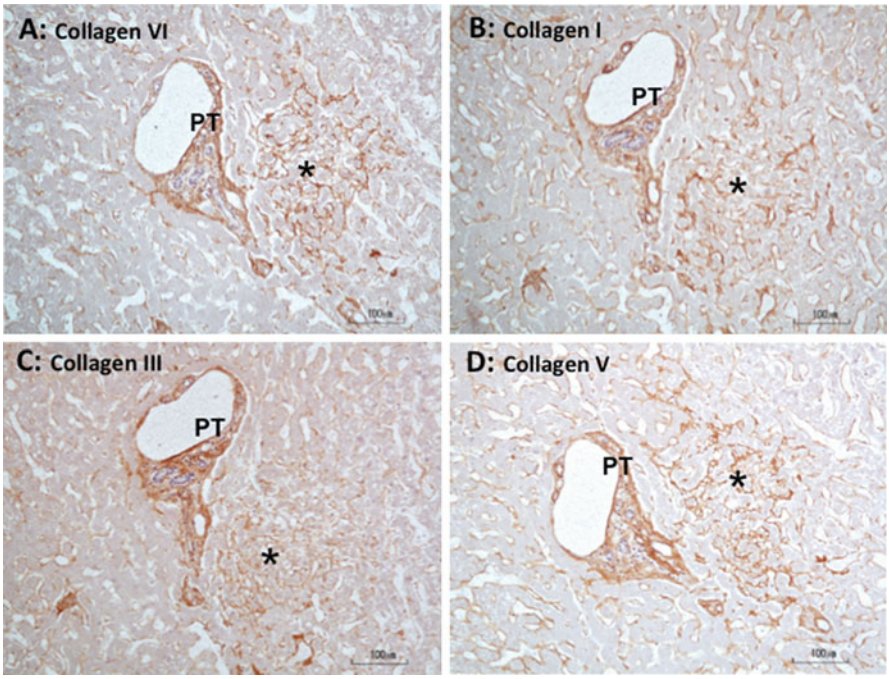

Fig. 6 Codistribution of collagens VI with collagens I, III, V in liver fibrosis. Codistribution of collagens VI, I, III, and V in fibrotic lesion of cadaveric liver. The tissue from embalmed elderly cadavers was processed for paraffin embedment cadavers as previously described (Mak et al. 2012), and immunoperoxidase staining was performed as indicated in Fig. 4. Collagen I antibody was rabbit polyclonal obtained from Rockland Immunochemicals (Gilbertsville, PA), and rabbit polyclonal collagen VI antibody was from Novus Biologicals (Littleton, CO). (**a**–**d**) are serial sections (5 μm thick) stained for collagens VI, I, III, and V, respectively. (**a**) The *asterisk* marks an area with perisinusoidal fibrosis in the periportal parenchyma, showing increased collagen VI immunostaining (*brown*) compared to the staining reaction surrounding the lesion. The increased collagen VI staining is coincident with enhanced staining for collagen I (**b**), collagen III (**c**), and collagen V (**d**) in the same fibrotic lesion, demonstrating co-distribution of these collagens. Hematoxylin counterstained. *PT* portal tract

Indeed, in vitro assays revealed that the degrees of MMP binding to the α2(VI) chain correlate with the inhibition of enzymatic activities of the MMPs. The binding of MMPs to collagen VI involves specifically the α2(VI) chain. Thus, it was proposed that collagen VI, which is upregulated in liver fibrosis, serves as a reservoir for the latent proMMPs and that the α2(VI) chain, as a binding molecule of proMMP-1, proMMP-3, and proMMP-8, modulates the availability and activities of the MMPs by sequestering the proteinases in the ECM of the fibrotic liver. Collagen VI binding of MMPs likely conserves the proform configuration of MMPs and protects these enzymes from activation, thereby diminishing matrix turnover and fibrolysis. Consequently, this biological action may perpetuate fibrous tissue deposition in the liver matrix, resulting in the progression of fibrogenesis.

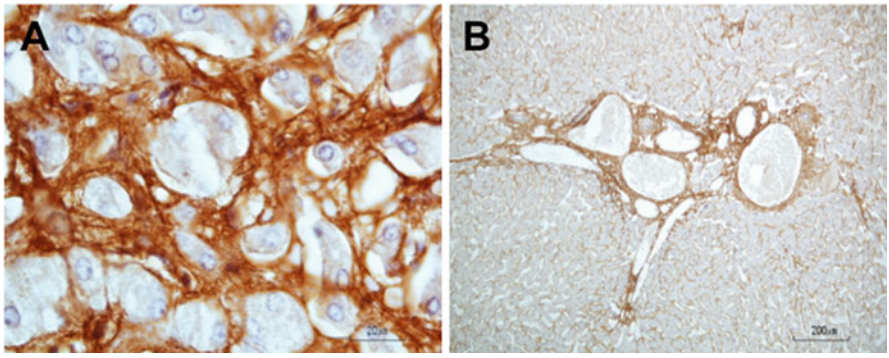

Fig. 7 Immunoperoxidase staining for type Vl collagen in liver fibrosis. (**a**) This high magnification image illustrates immunostaining for collagen VI in a severe perisinusoidal/pericellular fibrosis, described as chicken-wire fibrosis. The collagen VI immunostain (*brown*) in the lesion is robust and could be discerned as fibrils, forming a mesh around the hepatocytes. (**b**) Portal tract fibrosis. The fibrotic portal tract, characterized by irregular border with emerging short septa, is strongly stained for collagen V in the stroma. Hematoxylin counterstained

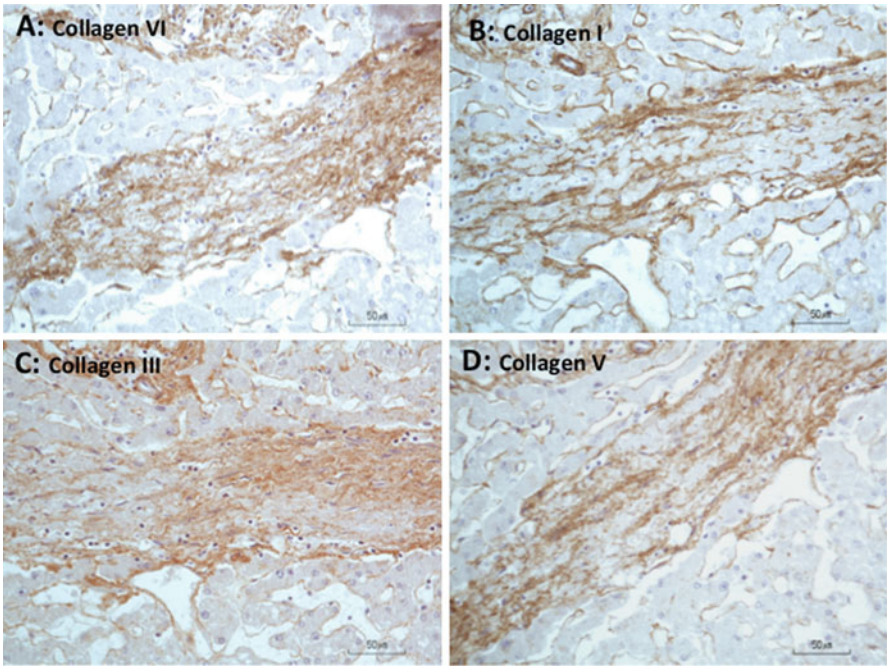

Fig. 8 Codistribution of collagens VI, I, III, and V in fibrous septum. Immunoperoxidase staining of fibrous septum in elderly cadaveric liver with bridging fibrosis. (**a**–**d**) are serial sections (5 μm thick) of a septum stained for collagens VI, I, III, and V, respectively. The matrix of the septum is positively stained (*brown*) for collagens VI, I, III, and V, thus demonstrating co-distribution of these proteins. Hematoxylin counterstained (From Mak et al. 2014, Austin Biomarkers and Diagnosis 1(2): id1012, 2014)

HSC Collagen VI Receptor as Antifibrotic Drug Target

The perisinusoidal HSCs are the principal ECM-producing cells of the liver. HSCs become activated in response to fibrogenic stimuli and produce increased amounts of ECM, particularly fibrillar collagens and possibly the filamentous collagen VI. Therefore, collagen VI cell surface receptors expressed on HSCs are attractive targets for antifibrotic agents. Because the cyclic octapeptide (C*GRGDSPC*) containing the RGD sequence Arg-Gly-Asp specifically binds to mesenchymal cells via type VI collagen receptors (Marcelino and McDevitt 1995), it was used to design a specific carrier targeting HSCs in the liver. To that effect, the cyclic peptide was covalently coupled to human serum albumin (HSA), yielding pCVI-HSA (Beljaars et al. 2000). Accordingly, the distribution of pCVI-HSA in normal and in bile duct ligation-induced fibrotic rat livers was evaluated. There was a preferential distribution of pCVI-HSA to the control normal livers and the fibrotic livers (62–75% of the total dose) at 10 min after an intravenous injection. Immunohistochemical analysis, however, revealed that 73% of the injected dose of pCVI-HSA predominantly localized to HSCs in the fibrotic liver. Importantly, in vitro studies showed that pCVI-HSA specifically bound to culture-activated HSCs and was internalized by these cells. Therefore, pCVI-HSA targeting activation-induced cell receptors may be employed as a carrier to deliver antifibrotic agents or drugs to HSCs to enhance the effectiveness and tissue selectivity of these factors against fibrogenesis. These findings highlight the involvement of HSC-associated collagen VI receptors in the pathogenesis of liver fibrosis.

Indicator of Early Liver Fibrogenesis

In the normal human liver, the interstitial fibrillar collagens I and III represent the most abundant collagens in the ECM, while the amount of filamentous collagen VI is low, accounting for less than 0.1% of total hepatic collagens (Schuppan 1990; Schuppan et al. 1985). Elevated serum concentrations of collagen VI occur in chronic liver fibrotic disease irrespective of the underlying causes of liver damage, including viral hepatitis, schistosomiasis infection, children with cystic fibrosis, and alcoholic cirrhosis (Shahin et al. 1992; Gerling et al. 1997; Stickel et al. 2001). It was proposed that collagen VI serves as a predictor of liver fibrosis. Strikingly, circulating levels of collagen VI are already raised in the early stages of alcoholic liver injury (Stickel et al. 2001). Because serum collagen VI levels may reflect the activity of fibrolysis, its increase in the circulation likely represents an enhanced tissue turnover of collagen VI in the early events of hepatic fibrotic transformation and therefore is a good indicator of early fibrogenesis. In cirrhosis, tissue collagen VI levels rose tenfold compared to the control levels (Schuppan et al. 1985), while the serum concentrations of collagen VI almost doubled that of the control (Shahin et al. 1992; Gerling et al. 1997; Stickel et al. 2001). One hypothesis is that during the histogenesis of advanced fibrosis, degradation of collagen VI is impaired (Schuppan et al. 1985), resulting in a higher tissue concentration of collagen VI, possibly

sustaining fibrogenesis by stimulation of activated HSCs or myofibroblasts for ECM production.

Biomarker for Hepatic Fibrosis

Despite advances made in the understanding of collagen VI's involvement in liver fibrosis and progression to cirrhosis, the issue of collagen VI serving as a liver fibrosis biomarker has yet to be addressed. In a number of authoritative reviews of liver fibrosis biomarkers, collagen VI has not been included in the discussion as a fibrosis marker (Gressner et al. 2007; Fallatah 2014). This may have been overlooked due to the lesser abundance of collagen VI relative to collagens I, III, and IV in the liver ECM.

Neo-epitope and Protein Fingerprinting Technology

The development of collagen VI as a biomarker of hepatic fibrosis largely stemmed from the notion of neo-epitope generation in conjunction with protein fingerprinting technology. It is known that a highly regulated equilibrium between the synthesis and degradation of ECM proteins—particularly the collagen types—is required to achieve tissue homeostasis. A disruption of this equilibrium is regarded as the basis of pathological processes, fibrosis included. The degradation products of ECM proteins can be measured in biological fluids, and such measurement can give an indication of the disease activity and progression. MMPs and cysteine proteases are capable of degrading collagens and proteoglycans of the ECM, respectively, which result in the generation of specific cleavage peptide fragments, called neo-epitopes (Karsdal et al. 2009). Because the generation of neo-epitopes occurs locally in the pathologically affected areas and involves a specific disease, it may carry a unique disease peptide fingerprint. This approach has been given the term "protein fingerprinting technology" (Karsdal et al. 2011; Leeming et al. 2013; Schierwagen et al. 2013; Vassiliadis et al. 2013). Therefore, compared to measurement of the intact/whole proteins, quantifying neo-epitopes—or protein fingerprint—is likely to provide a more sensitive indicator of a pathological change. In particular, these neo-epitope fragments are small enough to be released into the circulation or urine, thereby allowing their detection by antibodies raised specifically to react against the neo-epitopes. This is commonly measured by ELISA—as schematized in Fig. 9. Thus, measurement of neo-epitopes in the serum may indicate the degree of remodeling of ECM collagens and proteins that are involved in the development of liver fibrosis. Notably, neo-epitopes have successfully been used as noninvasive serum markers in osteoporosis and arthritis (Karsdal et al. 2009), which are both characterized by extensive ECM remodeling.

Collagen VI Neo-epitope as a Biomarker of Experimental Liver Fibrosis

Collagen VI constitutes a minor component of the total hepatic collagen, but its level is significantly elevated in the event of liver fibrogenesis, as discussed above. To that effect, neo-epitopes derived from the cleavage peptide fragments of collagen VI

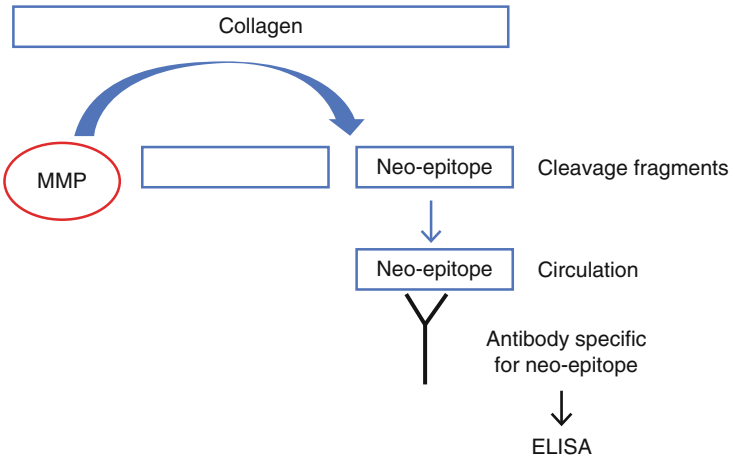

Fig. 9 Schema of enzyme-generated neo-epitope for ELISA. This diagram illustrates a specific matrix metalloproteinase (MMP) that acts on a selective collagen substrate, generating a peptide fragment – neo-epitope – which is released into the systemic circulation where it is detected by antibody specific for the neo-epitope. The antibody and neo-epitope reaction is quantified by ELISA

generated by the proteolytic action of MMP-2 or MMP-9 in vitro were used to raise antibodies that specifically react with the neo-epitopes, which can be quantified by ELISA (Veidal et al. 2011). Among the peptide fragments, the neo-epitope containing the sequence 573′.YRGPEGPQGPPG′584 in the α1 chain of collagen VI was selected based on the ability of the antibody to distinguish between the cleaved and uncleaved collagen VI. Also, this sequence was found to be 100% homologous to human, rat, and mouse and is designated as CO6-MMP. Accordingly, the value of CO6-MMP as a biomarker of liver fibrosis was evaluated in two rat models of hepatic fibrosis: bile duct ligation and carbon tetrachloride (CCl_4) treatment (Veidal et al. 2011). It was demonstrated that CO6-MMP serum concentrations were significantly elevated and were highly associated with the histological severity of liver fibrosis in these animals. Importantly, because the CO6-MMP antibody is capable of quantifying collagen VI degradation by MMP-2 and MMP-9, it can be employed to assess collagen VI turnover in early stages of fibrogenesis, serving as an early marker for fibrosis, which is consistent with the conclusion of previous studies that collagen VI is a good indicator of early fibrogenesis (Stickel et al. 2001). It has yet to be determined whether or not MMP-2 and MMP-9 degraded collagen VI represents a useful biomarker for the clinical assessment of liver fibrogenesis.

In another study by Leeming et al. (2013), hepatic collagen in CCl_4-treated rats was quantified by histomorphometry of Sirius red staining for collagens, and the values were expressed as four quartiles Q1, Q2, Q3, and Q4, representing early, moderate, severe fibrosis, and cirrhosis, respectively. Levels of serum CO6-MMP neo-epitope—also designated as C6M—were significantly elevated in all collagen quartiles in CCl_4-treated rats compared to controls. When evaluated as a single

collagen VI degradation marker, CO6-MMP cannot distinguish early, moderate, or severe fibrosis; however, when CO6-MMP was used in conjunction with P5CP, which is a collagen V formation marker, the combination of the two markers showed the highest and best correlation with total hepatic collagens in all quartiles than any single marker, including that of collagen I, III, or IV. The combination of scores generally enabled separation of early fibrosis, severe fibrosis, and cirrhosis from the respective controls. Moreover, the combined scores differentiate early and moderate fibrosis, as well as severe fibrosis and cirrhosis. Hence, the combined use of collagen VI and collagen V biomarkers is the most reliable indicator of both early- and late-stage fibrosis.

Conclusions

The molecular structure of type VI collagen has largely been determined since its discovery 32 years ago, and significant advances are being made in the fields of collagen VI-related muscular disorders, mammary carcinogenesis, and fibrotic diseases such as adipose tissue and liver fibrosis. There are clinical data pointing to collagen VI as a marker indicative of early hepatic fibrotic changes in alcoholic patients. Experimental data indicate that the collagen VI receptor expressed on HSCs offers a selective target for antifibrotic agents, but this area has so far been understudied. Studies using collagen VI knockout mice in conjunction with induction of fibrosis—CCl_4 treatment or bile duct ligation—could help determine whether collagen VI plays a specific role in liver fibrogenesis. Collagen VI-derived neo-epitope (CO6-MMP), which can be quantified by ELISA, is useful in assessing tissue turnover in early fibrogenesis. Furthermore, the combined use of neo-epitopes of collagen VI and collagen V offers the most reliable indicator of both early- and late-stage fibrosis; however, its application as a noninvasive serum biomarker in patients has yet to be determined because the pathogenesis of fibrosis in humans is likely to be more variable than in the CCl_4 fibrosis model.

Potential Applications to Prognosis, Other Diseases, or Conditions

C06-MMP, the neo-epitope of collagen VI generated by the proteolytic degradation of MMP-2 and MMP-9-during liver fibrogenesis, serves as a useful noninvasive biomarker in two models of experimental liver fibrosis. Its increased presence in the serum is highly associated with liver fibrogenesis, reflecting the central role of VI collagen turnover in ECM remodeling in fibrogenesis. It is, however, regarded only as an investigative marker in accordance with the BIPED (burden of disease, investigative, prognostic, efficacy of intervention, and diagnostic) system (Liu et al. 2012). Accordingly, further studies are needed to evaluate whether

CO6-MMP has any potential applications to prognosis of hepatic fibrotic disease. It is equally important to assess whether CO6-MMP—either alone or in combination with other collagen type neo-epitopes–can be employed to monitor the efficacy of antifibrotic agents in the therapy of liver fibrosis or intervention of fibrosis progression. Because liver fibrosis and even cirrhosis can be reversed by eliminating the underlying cause of the disease, or upon cessation of the fibrogenic stimulant in the CCl_4 reversible model of fibrosis, it is clinically relevant to investigate whether CO6-MMP is useful for monitoring fibrosis regression. Finally, because collagen VI is involved and upregulated in other fibrotic diseases such as adipose tissue, heart, kidneys, lungs, and skin (Mak et al. 2014), CO6-MMP may serve as a potential biomarker for monitoring fibrotic changes in these organs.

Summary Points

- Type VI collagen is a filamentous collagen present in most connective tissue matrices where it forms a flexible network, linking matrix molecules and cells.
- Type VI collagen is composed of three genetically distinct chains, α1(VI), α2(VI), and α3(VI), with a globular domain at each end.
- In the cells, collagen VI monomers dimerize and form tetramers, which are secreted and associate into filaments in the extracellular matrix.
- Collagen VI gene expression is regulated differently than collagen I or III. Collagen VI interacts with collagen V and fibronectin, contributing to the structurally integrity of tissue matrix scaffolds.
- Collagen VI mediates cell adhesion and promotes migration, and soluble collagen VI acts as a sensor for tissue damage, modulating mesenchymal cell proliferation and survival, matrix homeostasis, and wound healing.
- Three collagen VI-deficient mouse models have been generated, which have been used to investigate collagen VI-related myopathies, mammary tumorigenesis, and skeletal muscle satellite cell homeostasis.
- Collagen VI expression is upregulated in fibrosis of adipose tissue and liver.
- Elevated collagen VI in circulation is considered an early biomarker of alcoholic liver fibrosis.
- Collagen VI immunostaining is enhanced in fibrotic foci, codistributing with collagens I, III, and V. Hepatic stellate cells (HSCs) are likely the source of perisinusoidal collagen VI.
- The α2(VI) chain sequesters hepatic matrix metalloproteinase (MMP)-1, MMP-3, and MMP-8 and prevents the enzymes' activation, diminishing fibrolysis.
- The collagen VI receptor on HSCs offers selective targets for antifibrotic agents.
- CO6-MMP, a collagen VI neo-epitope generated by the proteolytic actions of MMP-2 or MMP-9, serves as a specific biomarker of collagen VI degradation in experimental liver fibrogenesis.

References

Amenta PS, Gil J, Martinez-Hernandez A. Connective tissue of rat lung. II. Ultrastructural localization of collagen types III, IV, and VI. J Histochem Cytochem. 1988;36:1167–73.

Arthur MJP. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. Am J Physiol Gastrointest Liver Physiol. 2000;279:245–9. Collagenases and liver fibrosis.

Atkinson JC, Rühl M, Becker J, et al. Collagen VI regulates normal and transformed mesenchymal cell proliferation in vitro. Exp Cell Res. 1996;228:283–91.

Baldock C, Sherratt MJ, Shuttleworth CA, et al. The supramolecular organization of collagen VI microfibrils. J Mol Biol. 2003;330:297–307.

Beljaars L, Molema G, Schuppan D, et al. Successful targeting to rat hepatic stellate cells using albumin modified with cyclic peptides that recognize the collagen type VI receptor. J Biol Chem. 2000;275:12745–51.

Bonaldo P, Braghetta P, Zanetti M, et al. Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy. Hum Mol Genet. 1998; 7: 2135–40.

Bruns RR, Press W, Engvall E, et al. Type VI collagen in extracellular, 100-nm periodic filaments and fibrils: identification by immunoelectron microscopy. J Cell Biol. 1986;103:393–4.

Chu M-L, Conway D, Pan T, et al. Amino acid sequence of the triple-helical domain of human collagen type VI. J Biol Chem. 1988;263:18601–6.

Colombatti A, Bonaldo P. Biosynthesis of chick type VI collagen. II. Processing and secretion in fibroblasts and smooth muscle cells. J Biol Chem. 1987;262:14461–6.

Colombatti A, Bonaldo P, Ainger K, et al. Biosynthesis of chick type VI collagen. I. Intracellular assembly and molecular structure. J Biol Chem. 1987;262:14454–60.

Colombatti A, Mucignat MT, Bonaldo P. Secretion and matrix assembly of recombinant type VI collagen. J Biol Chem. 1995;270:13105–11.

Engel J, Furthmayr H, Odermatt E, et al. Structure and macromolecular organization of type VI collagen. Ann NY Acad Sci. 1985;460:25–37.

Engvall E, Hessle H, Klier G. Molecular assembly, secretion, and matrix deposition of type VI collagen. J Cell Biol. 1986;102:703–10.

Everts V, Korper W, Niehof A, et al. Type VI collagen is phagocytosed by fibroblasts and digested in the lysosomal apparatus: involvement of collagenase, serine proteinases and lysosomal enzymes. Matrix Biol. 1995;14:665–76.

Fallatah HI. Noninvasive biomarkers of liver fibrosis: an overview. Hindawi Publishing Corp. Adv Hepatol. 2014, Article ID 357287, 15 pages.

Fitzgerald J, Rich C, Zhou FH, et al. Three novel collagen VI chains, α4(VI), α5(VI), and α6(VI). J Biol Chem. 2008;283:20170–80.

Freise C, Erben U, Muche M, et al. The alpha 2 chain of collagen type VI sequesters latent proforms of matrix metalloproteinases and modulates their activation and activity. Matrix Biol. 2009;28:480–9.

Furthmayr H, Wiedemann H, Timpl R, et al. Electron-microscopical approach to a structural model of intima collagen. Biochem J. 1983;211:303–11.

Gara SK, Grumati P, Urciuolo A, et al. Three novel collagen VI chains with high homology to the α3 chain. J Biol Chem. 2008;283:10658–70.

Gara SK, Grumati P, Squarzoni S, et al. Differential and restricted expression of novel collagen VI chains in mouse. Matrix Biol. 2011;30:248–57.

Gerling B, Becker M, Stabb D, et al. Prediction of liver fibrosis according to serum collagen level in children with cystic fibrosis. N Engl J Med. 1997;336:1611–2.

Gressner OA, Weiskirchen R, Gressner AM. Biomarkers of liver fibrosis: clinical translation of molecular pathogenesis or based on liver-dependent malfunction tests. Clin Chim Acta. 2007;381:107–13.

Griffiths MR, Shepherd M, Ferrier R, et al. Light microscopic and ultrastructural distribution of type VI collagen in human liver: alterations in chronic biliary disease. Histopathology. 1992;21:335–44.

Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol. 1992;12:954–61.

Hatamochi A, Aumailley M, Mauch C, et al. Regulation of collagen VI expression in fibroblasts. Effects of cell density, cell-matrix interactions, and chemical transformation. J Biol Chem. 1989;264:3494–9.

Heckmann M, Aumailley M, Chu M-L, et al. Effect of transforming growth factor-β on collagen type VI expression in human dermal fibroblasts. FEBS Lett. 1992;310:79–82.

Hicks D, Lampe AK, Barresi R, et al. A refined diagnostic algorithm for Bethlem myopathy. Neurology. 2008;70:1192–9.

Howell SJ, Doane KJ. Type VI collagen increases cell survival and prevents anti-β1integrin-mediated apoptosis. Exp Cell Res. 1998;241:230–41.

Iyengar P, Espina V, Williams TW, et al. Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/stroma microenvironment. J Clin Invest. 2005;115:1163–76.

Karsdal MA, Henriksen K, Leeming DJ, et al. Biochemical markers and the FDA critical path: how biomarkers may contribute to the understanding of pathophysiology and provide unique and necessary tools for drug development. Biomarkers. 2009;14:181–202.

Karsdal MA, Delvin E, Christiansen C. Editorial protein fingerprints – relying on and understanding the information of serological protein measurements. Clin Biochem. 2011;44:1278–9.

Keene DR, Engvall E, Glanville RW. Ultrastructure of type VI collagen in human skin and cartilage suggests an anchoring function for this filamentous network. J Cell Biol. 1988;107:1995–2006.

Khan T, Muise ES, Iyengar P, et al. Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. Mol Cell Biol. 2009;29:1575–91.

Kielty CM, Lees M, Shuttleworth A, et al. Catabolism of intact type VI collagen microfibrils: susceptibility to degradation by serine proteinases. Biochem Biophys Res Commun. 1993;191:1230–6.

Knupp C, Squire JM. Molecular packing in network-forming collagens. Adv Protein Chem. 2005;70:375–403.

Kobayasi T, Karismark T. Type V and VI collagen for cohesion of dermal structures. J Submicrosc Cyto Pathol. 2006;38:103–8.

Kuo HJ, Cheryl L, Maslen CL, et al. Type IV collagen anchors endothelial basement membranes by interacting with type VI collagen. J Biol Chem. 1997;272:26522–9.

Leeming DJ, Byrjalsen I, Jimenez W, et al. Protein fingerprinting of the extracellular matrix remodelling in a rat model of liver fibrosis – a serological evaluation. Liver Int. 2013;33:439–47.

Liu T, Wang X, Karsdal MA, et al. Molecular serum markers of liver fibrosis. Biomark Insights. 2012;7:105–17.

Loreal O, Clement B, Schuppan D, et al. Distribution and cellular origin of collagen VI during development and in cirrhosis. Gastroenterology. 1992;102:980–7.

Mak KM, Kwong AJ, Chu E, et al. Hepatic steatosis, fibrosis, and cancer in elderly cadavers. Anat Rec. 2012;295:40–50.

Mak KM, Sehgal P, Harris CK. Type VI collagen: its biology and value as a biomarker of hepatic fibrosis. Austin Biomark Diagn. 2014;1:9.

Marcelino J, McDevitt CA. Attachment of articular cartilage chondrocytes to the tissue form of type VI collagen. Biochim Biophys Acta. 1995;1249:180–8.

Martinez-Hernandez A, Amenta PS. The hepatic extracellular matrix. II. Ontogenesis, regeneration and cirrhosis. Virchows Arch A Pathol Anat Histopathol. 1993;423:77–84.

McCulloch LJ, Rawling TJ, Sjoholm K, et al. COL6A3 is regulated by leptin in human adipose tissue and reduced in obesity. Endocrinology. 2015;156:134–46.

Myint E, Brown DJ, Ljubimov AV, et al. Cleavage of human corneal type VI collagen α3 chain by matrix metalloproteinase-2. Cornea. 1996;15:490–6.

Pan TC, Zhang RZ, Markova D, et al. COL6A3 protein deficiency in mice leads to muscle and tendon defects similar to human collagen VI congenital muscular dystrophy. J Biol Chem. 2013;288:14320–31.

Pan TC, Zhang RZ, Arita M, et al. A mouse model for dominant collagen VI disorders: heterozygous deletion of Col6a3 exon 16. J Biol Chem. 2014;289:10293–307.

Park J, Scherer PE. Adipocyte-derived endotrophin promotes malignant tumor progression. J Clin Invest. 2012;122:4243–56.

Pasarica M, Gowronska-Kozak B, Burk D, et al. Adipose tissue collagen VI obesity. J Clin Endocrinol Metab. 2009;94:5155–62.

Ruehl M, Wiecher D, Sahin E, et al. Soluble collagen VI as an auto paracrine inhibitor of apoptosis in hepatic stellate cells. Gastroenterology. 1999;116:10392.

Rühl M, Johannsen M, Atkinson J, et al. Soluble collagen VI induces tyrosine phosphorylation of paxillin and focal adhesion kinase and activates the MAP kinase erk2 in fibroblasts. Exp Cell Res. 1999a;250:548–57.

Rühl M, Sahin E, Johannsen M, et al. Soluble collagen VI drives serum-starved fibroblasts through S- phase and prevents apoptosis via down-regulation of Bax. J Biol Chem. 1999b;274:34361–8.

Sabatelli P, Bonaldob P, Lattanzia G, et al. Collagen VI deficiency affects the organization of fibronectin in the extracellular matrix of cultured fibroblasts. Matrix Biol. 2001;20:475–86.

Sabatelli P, Gara SK, Grumati P, et al. Expression of the collagen VI α5 and α6 chains in normal human skin and in skin of patients with collagen VI-related myopathies. J Invest Dermatol. 2011;131:99–107.

Scherer PE, Bickel PE, Kotler M, et al. Cloning of cell-specific secreted and surface proteins by subtractive antibody screening. Nat Biotechnol. 1998;16:581–6.

Schierwagen R, Leeming DJ, Klein S, et al. Biomarkers of liver fibrosis: clinical translation of molecular pathogenesis or based on liver-dependent malfunction tests. Front Physiol. 2013;4:1–9, Article 195. doi:103389/fphys.2013.00195.

Schuppan D. Structure of the extracellular matrix in normal and fibrotic liver; collagens and glycoproteins. Semin Liver Dis. 1990;10:1–10.

Schuppan D, Ruhlmann T, Hahn EG. Radioimmunoassay for human type VI collagen and its application to tissue and body fluids. Anal Biochem. 1985;149:238–47.

Schuppan D, Ruehl M, Somasundaram R, et al. Matrix as a modulator of hepatic fibrogenesis. Semin Liver Dis. 2001;21:351–72.

Shahin M, Schuppan D, Waldherr R, et al. Serum procollagen peptides and collagen type VI for the assessment of activity and degree of hepatic fibrosis in schistosomiasis and alcoholic liver disease. Hepatology. 1992;15:637–44.

Somasundaram R, Schuppan D. Type I, II, III, IV, V, and VI collagens serve as extracellular ligands for the isoforms of platelet-derived growth factor (AA, BB, and AB). J Biol Chem. 1996;271:26884–91.

Somasundaram R, Ruehl M, Schaefer B, et al. Interstitial collagens I, III, and VI sequester and modulate the multifunctional cytokine oncostatin M. J Biol Chem. 2002;277:3242–6.

Spencer M, Yao-Borengasser A, Unal R, et al. Adipose tissue macrophages in insulin-resistant subjects are associated with collagen VI and fibrosis and demonstrate alternative activation. Am J Physiol Endocrinol Metab. 2010;299:E1016–27.

Stickel F, Urbaschek R, Schuppan D, et al. Serum collagen type VI and XIV and hyaluronic acid as early indicators for altered connective tissue turnover in alcoholic liver disease. Dig Dis Sci. 2001;46:2025–32.

Sun K, Park J, Gupta OT, et al. Endotrophin triggers adipose tissue fibrosis and metabolic dysfunction. Nat Commun. 2014;5:3485.

Takahara T, Sollberg S, Muona P, et al. Type VI collagen gene expression in experimental liver fibrosis: quantitation and spatial distribution of mRNAs, and immunodetection of the protein. Liver. 1995;15:78–86.

Tillet E, Ruggiero F, Nishiyama A, et al. The membrane-spanning proteoglycan NG2 binds to collagens V and VI through the central nonglobular domain of its core protein. J Biol Chem. 1997;272:10769–76.

Urciuolo A, Quarta M, Morbidoni V, et al. Collagen VI regulates satellite cell self-renewal and muscle regeneration. Nat Commun. 2013;4:1964.

Vassiliadis E, Barascuk N, Karsdal MA. Atherofibrosis – a unique and common process of the disease pathogenesis of atherosclerosis and fibrosis – lesions for biomarker development. Am J Transl Res. 2013;5:1–14.

Veidal SS, Karsdal MA, Vassiliadis E, et al. MMP mediated degradation of type VI collagen is highly associated with liver fibrosis – identification and validation of a novel biochemical marker assay. PLoS One. 2011;6:e24753.

Von Der Mark H, Aumailley M, Wick G, et al. Immunohistochemistry, genuine size and tissue localization of collagen VI. Eur J Biochem. 1984;142:493–502.

Weil D, Mattei MG, Passage E, et al. Cloning and chromosomal localization of human genes encoding the three chains of type VI collagen. Am Hum Genet. 1988;42:435–45.

Hydroxyproline as a Biomarker in Liver Disease

22

Sami A. Gabr, Ahmad H. Alghadir, Yousery E. Sherif, and Ayman A. Ghfar

Contents

S.A. Gabr (✉)
Department of Rehabilitation Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Riyadh, Saudi Arabia

Department of Anatomy, Faculty of Medicine, Mansoura University, Mansoura, Egypt
e-mail: nadalab2009@hotmail.com; drGabr14@yahoo.com; sgabr@ksu.edu.sa

A.H. Alghadir
Department of Rehabilitation Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Riyadh, Saudi Arabia
e-mail: aalghadir@hotmail.com

Y.E. Sherif
Department of Chemistry, Faculty of Science and Arts, Ulla, Taibah University, Medina, Saudi Arabia

Clinical Pharmacology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt
e-mail: youserysherif@hotmail.com

A.A. Ghfar
Department of Chemistry, College of Science, King Saud University, Riyadh, Saudi Arabia
e-mail: aymanghfar@gmail.com

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_26

Abstract

Hepatic fibrosis is one of the most critical diseases of wounded tissues that arise from different caustic agents, especially viral hepatitis, bilharzia, chemical- and drug-induced toxicity, and metabolic disorders. The proceeding of fibrosis depends mainly up on imbalance between the rate of formation and deposition of collagen which associated with many metabolic and biochemical abnormalities.

Hydroxyproline is one of the most amino acids present in collagen following hydroxylation of proline moiety. The presence of hydroxyproline in extracellular matrix (ECM) produced by activated hepatic stellate cells (HSCs) preserves the integrity and function of liver cells. Its level in liver tissues, serum, and urine comprises a superior limiting factor which could signify correctly the rates and progression of liver fibrogenesis. The estimation of hydroxyproline in different research studies as marker for diagnosis or measurement of the anti-fibrotic activity of therapeutic interventions of herbal or non-herbal medicine argues its importance as noninvasive fibrotic detecting biomarker in chronic liver diseases with severe fibrosis.

Keywords

Hydroxyproline • Liver fibrosis • Viral hepatitis • Toxicity • Herbal medicine • Collagen • Hepatic stellate cells (HSCs)

List of Abbreviations

AUROCs	Area under receiver operating characteristic curves
BMP-7	Bone morphogenetic protein-7
CCA	Cholangiocarcinoma
CCL_4 toxicity	Carbon tetrachloride toxicity
CHB	Chronic hepatitis B
DMN	Dimethylnitrosamine
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
GSH	Glutathione reduced form
GSSG	Glutathione oxidized form
HBD	Hepatobiliary diseases
HCV	Hepatitis C virus
HPLC	High-performance liquid electrophoresis
HSCs	Hepatic stellate cells
HSS	Hypersplenomegaly syndrome
HYP	Hydroxyproline
MAP	Mitogen-activated protein
MAT1A; MAT2A	Methionine adenosyltransferase 1, 2
MDA	Malondialdehyde
MET	Mesenchymal-to-epithelial transition
MSS	Moderate splenomegaly syndrome

PDGF	The platelet-derived growth factor
TAA toxicity	Thioacetamide toxicity
TGF-β1	Transforming growth factor β1
YGJD	Yi Guan Jian Decoction
α-SMA	α-Smooth muscle actin

Key Facts of Liver Fibrosis

- The prognosis of chronic liver diseases depends mainly on the degree of liver fibrosis.
- The deposition and degradation of collagen protein within liver tissues closely linked with the severity of liver fibrosis.
- Advanced stages of liver fibrosis were shown to be linked with both liver dysfunction and cellular architectural changes.
- Histological assessment of fibrosis using liver biopsy comprises the gold standard method for estimation of the severity of liver diseases.
- Liver biopsy is regarded as the benchmark for validation of noninvasive techniques to measure noninvasive reliable biomarkers for diagnosing and grading hepatic fibrosis especially in anti-fibrotic interventions of clinical interest.

Definitions of Words and Terms

Collagen	It is the main structural protein in the extracellular space in the various connective tissues in animals. As the main component of connective tissue, it is the most abundant protein in mammals, making up from 25% to 35% of the whole-body protein content.
Extracellular matrix (ECM)	It is a collection of extracellular molecules secreted by cells that provides structural and biochemical support to the surrounding cells. Cell adhesion and cell-to-cell communication and differentiation are common functions of the ECM.
Fibrosis	It is a reactive, benign, or pathological state of the formation of excess fibrous connective tissue in an organ or tissue in a reparative or reactive process to maintain structure integrity and function of cells. It is used as a marker for diagnosing pathological cases.
Hepatic stellate cells (HSCs)	They have different names, and they are known as perisinusoidal cells or Ito cells (earlier lipocytes or fat-storing cells) or the space of Disse. The stellate

	cell is the major cell type involved in liver fibrosis, which is the formation of scar tissue in response to liver damage.
Hepatobiliary diseases (HBD)	They include a heterogeneous group of diseases of the liver and biliary system caused by viral, bacterial, and parasitic infections, neoplasia, toxic chemicals, alcohol consumption, poor nutrition, and metabolic disorders.
Herbal medicine	It is a type of nondrug modulators using phytoconstituents extracted from plants for medical treatments through much of human history.
High-performance liquid chromatography (HPLC)	It is formerly referred to as high-pressure liquid chromatography and is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material.
Hydroxyproline	It is a major component of the protein collagen produced from hydroxylation of proline amino acid. It plays a key role for collagen stability.

Introduction

Human body contains a complex extracellular matrix in organs and tissues that plays an integral part in their structure integrity and function. The most abundant of the extracellular matrix proteins are the collagens. In tissues, most of the matrix structure contains higher contents of collagens which control both synthetic and degradative processes. Thus, the imbalance between these processes affects upon the deposition rates of collagens within tissues and organs especially the lung, liver, kidney, and heart, resulting in fibrogenesis (White et al. 2003; Bedossa and Paradis 2003; Desmouliere et al. 2003).

Hydroxyproline is a non-proteinogenic amino acid that plays a pivotal role in maintaining cell structure and function in plant, animal, and human cells (Wu et al. 2010). It is formed by posttranslational hydroxylation of proline (Gordon and Hahn 2010). Both proline and hydroxyproline designate about one third of the amino acids reported in the collagen synthesis which comprises 30% of body proteins (Hu et al. 2008; Kaul et al. 2008). However, hydroxyproline constitutes about 4–13% of the amino acid content of various collagen forms. It has a major role in stabilizing the helical structure of collagen fibers (Nelson and Cox 2005; Gorres and Raines 2010).

Chemical Structures of Proline and Hydroxyproline

Due to the presence of α-amino group, proline and hydroxyproline are characterized as α-amino acids. The chemical structure of collagen proteins which exists in all connective tissues contains three chains of polypeptides (two α1 chains and one α2 chain), and the major part of these chains included proline and hydroxyproline amino acids (Krane 2008; Wu et al. 2010).

During formation of collagen triple helices, prolyl 4-hydroxylase catalyzes the formation of 4-hydroxyproline in collagen by the hydroxylation of proline residues in Gly-X-Pro-Gly-X-Y sequences. The reaction products, 4-hydroxyproline residues, serve to stabilize the helices (Pihlajaniemi et al. 1991), whereas proline can be in the X or Y position and hydroxyproline occurs only in the Y position of the repeating Gly-X-Y sequences (Krane 2008). The unique ring structure of proline and hydroxyproline distinguishes them from other AA in terms of rigidity, chemical stability, and biochemical reactions (Fig. 1).

Hydroxyproline and Liver Fibrosis

Granulation of liver tissues and activation of fibrosis depend mainly on the depositions of collagen fibers as a result of different causes (biological factors, chemo toxins, drugs, and autoimmune, parasitic, and viral infections) (Bruck et al. 2001). Chemically, collagen is composed of repeated units of three polypeptide chains containing glycine-proline amino acids. It is subscribed with over deposition of extracellular matrix (ECM), as a result of imbalance between synthesis and degradation of ECM in liver tissues (Wynn 2008). The stability of helical structures of collagen fibrils was maintained by deposition of hydroxyproline amino acid.

During formation of hydroxyproline, proline units were hydroxylated by proline hydroxylase enzyme in a catalytic hydroxylation manner using ascorbic acid and oxygen as cofactors (Drobnik and Dabrowski 1996; Tsuchiya and Bates 1997). Following prolonged hepatocyte damage, hepatic stellate cells (HSCs) were shown to play a distinguished role in liver fibrosis. During this process, HSC becomes activated and transformed from vitamin A-storing cell into proliferative and contractile myofibroblast-like cells, with overexpression of collagen fibers. The extent of HSC proliferation rates was denoted by expression of α-smooth muscle actin and the platelet-derived growth factor (PDGF) as new receptors (Kisseleva and Brenner 2007; Tacke and Weiskirchen 2012; Jiang and Torok 2013). Also, activated HSCs produce excessive amounts of extracellular matrix (ECM) proteins such as collagen types, proteoglycans, fibronectin, and laminin along with inhibitors of matrix metalloproteinase enzymes, resulting in an imbalance between fibrogenesis and fibrolysis and subsequent excessive deposition of matrix proteins. So, these cells have a significant role in progression of liver fibrosis stage to cirrhosis (Spira et al. 2002; Stalnikowitz and Weissbrod 2003; Lotersztajn et al. 2005). Finally, the excessive deposition of collagen

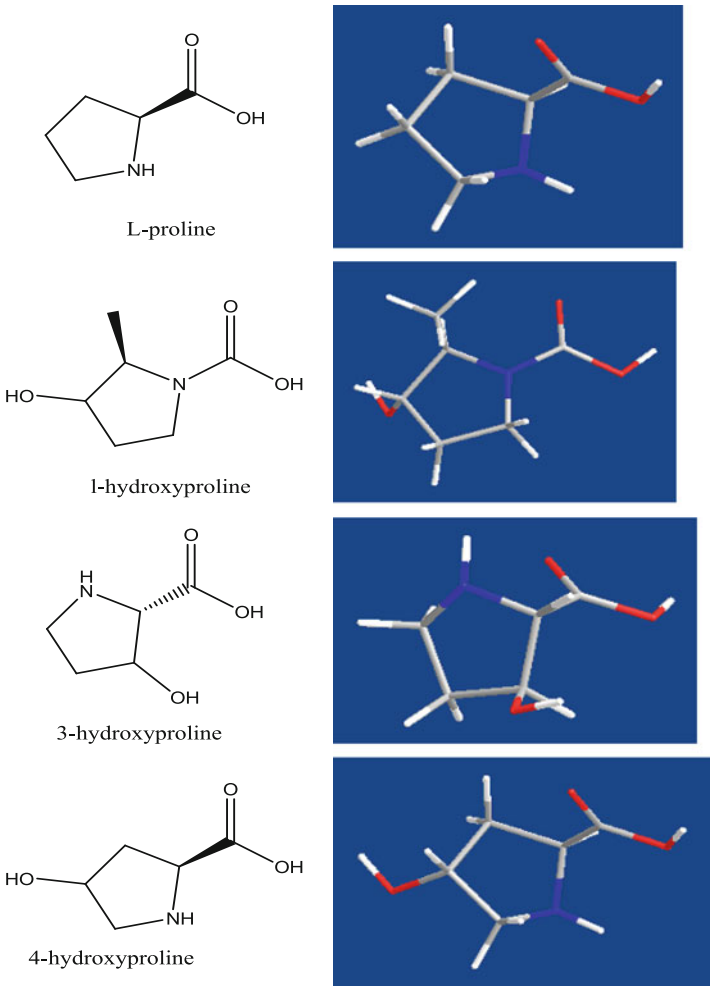

Fig. 1 Chemical structures of proline and hydroxyproline drawn with ChemOffice program (2008)

fibrils in liver connective tissues provides a hallmark of the development of liver fibrosis (Li and Friedman 1999; Friedman 2000) (Fig. 2). Thus, the measurement of collagen content or its amino acids in targets with liver diseases is considered as one of more reasonable practical aids for estimating the degree of liver fibrosis.

Hydroxyproline as a Marker in Liver Diseases

The development of fibrosis depends mainly on incorporation of proline into procollagen via posttranslational hydroxylation to hydroxyproline. The change in the content of these amino acids was shown to be significantly correlated with the

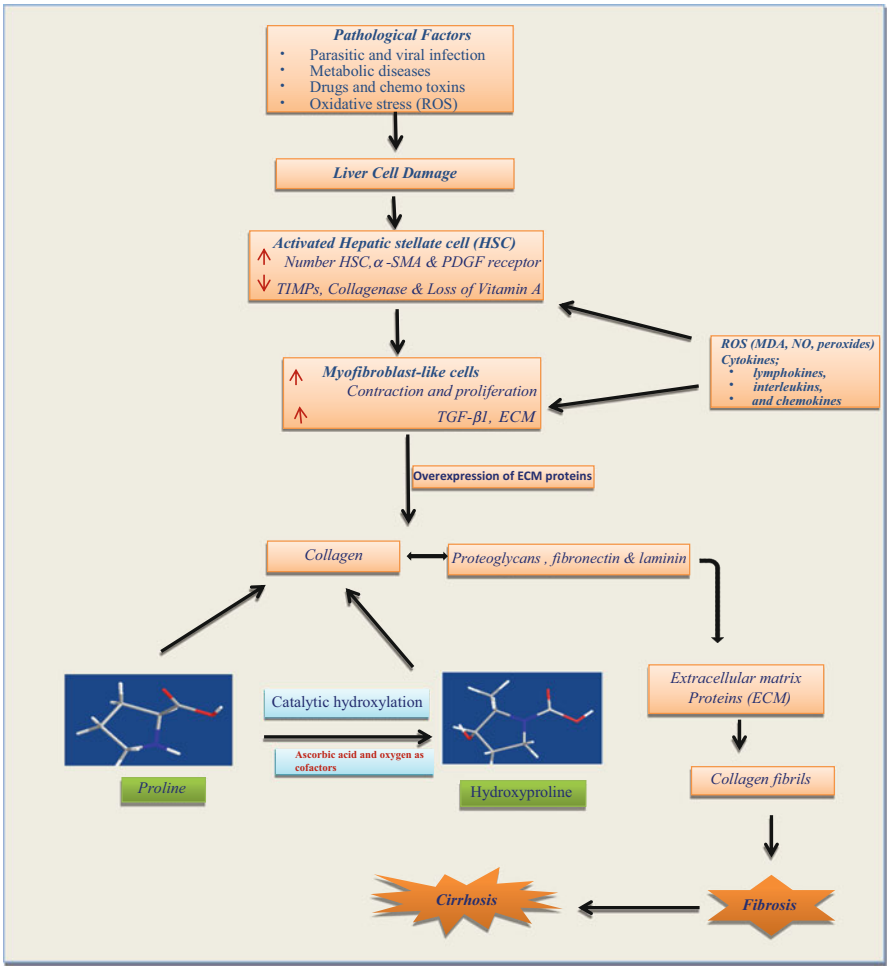

Fig. 2 Role of proline, hydroxyproline, and activated hepatic stellate cell (HSC) in liver fibrogenesis

rates of collagen during synthesis and degradation process and can be used in the assessment of collagen content in normal and fibrotic tissues. So, many methods should be evaluated to estimate the values of these amino acids as markers of collagen synthesis and degradation during the pathogenesis of fibrosis (McAnulty and Laurent 1987; McAnulty et al. 1991).

Based upon the rates of synthesis and degradation of collagen, many techniques such as radiolabeling (Prockop et al. 1961; Peterkofsky and Prockop 1962), HPLC (Lindblad and Diegelmann 1984; McAnulty et al. 1995), and colorimetric (Prockop and Udenfriend 1960) assays were evaluated to estimate the values of proline and hydroxyproline as a marker of collagen content. Also, the measurements of unlabeled moieties of free or low-molecular-weight hydroxyproline could be used

as valuable indices to compare with total hydroxyproline present in tissues during synthesis and degradation of collagen (Laurent and McAnulty 1983; McAnulty et al. 1988) as shown in Fig. 3.

The metabolism of hydroxyproline into equal amounts of pyruvate and glyoxylate originally occurs in hepatic mitochondria and renal proximal tubule cells (Phang et al. 2001; Knight and Holmes 2005). It was estimated that the daily turnover of collagen in humans is 2–3 g/day that leads to the metabolism of 240–420 mg of hydroxyproline and the formation of 140–240 mg of glyoxylate (Phang et al. 2001).

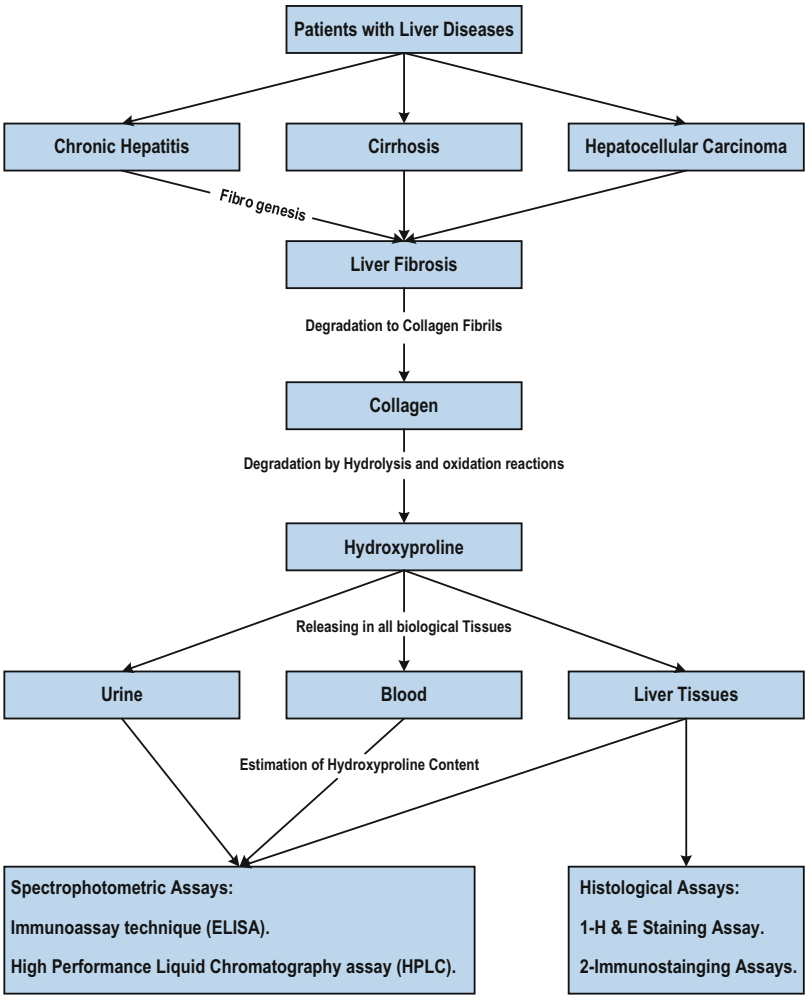

Fig. 3 Different techniques for hydroxyproline analysis from all biological tissues of patients with liver fibrosis

Hydroxyproline was shown to be the only unique amino acid that restricted for the synthesis of collagen fibrils in connective tissues. During collagen degradation, it was shown that the released hydroxyproline content in urine and serum significantly correlated with fibrosis, and it could be used as a diagnostic marker for fibrotic scores especially in bone and liver targets (Laitinen et al. 1966; Need 2006).

Thus, hydroxyproline provides a characteristic biochemical marker in tissue, serum, and urine samples that can simplify the chemical analysis of connective tissues (Adams and Frank 1980; Bedossa et al. 2003).

Hydroxyproline as a Fibrotic Marker in Urine

Three forms of hydroxyproline are present in urine: free, small, and nondialyzable polypeptides containing hydroxyproline. Only small hydroxyproline peptides comprise over 90% of urinary hydroxyproline excretion (Nobbs et al. 1975; Gasser et al. 1979).

The morbidity and mortality of HCV infection are significantly reported by the severity of liver fibrosis worldwide (Lauer and Walker 2001; Poynard et al. 1997). Thus, urinary hydroxyproline was one of the determinant markers of liver fibrosis among HCV patients subjected for drug therapy interventions of pegylated interferon 2a and ribavirin and telmisartan (Elsisi et al. 2012). The results of this study showed that the level of urinary hydroxyproline along with other serum fibrotic markers significantly correlated with Ishak fibrosis score of patient's liver biopsies and that the decrease in urinary hydroxyproline following drug therapy is considered as one of the valuable diagnostic markers which measures the anti-fibrotic efficiency of these drugs. Also in patients with chronic hepatitis B (CHB), urinary hydroxyproline was used as a marker for measuring of drug effect on hepatic fibrosis. The data showed significant increase in the levels of hydroxyproline in urine samples following Fuzheng Huayu 319 recipe treatment. The increase in hydroxyproline level may be due to the increase in degradation rates of collagen fibers (Ping et al. 1998).

In some animal experimental models, the development of hepatic fibrosis is accompanied with a number of biochemical changes. These lead to abnormalities in liver structure and change in the level of some metabolic products which released into blood and finally into urine. Urinary levels of hydroxyproline as a marker for liver fibrosis were estimated in rats with hepatic fibrosis induced by dimethylnitrosamine (DMN). The data of this study showed significant increases of hydroxyproline in serum along with the increase in the rate of its urinary excretion. This study suggested the importance of hydroxyproline as biochemical alterations during the pathogenesis of hepatic fibrosis (George and Chandrakasan 2000) and that there was extensive degradation of newly synthesized collagen in the liver during the early stages of fibrosis by collagenolytic enzymes (Murawaki et al. 1990).

Hydroxyproline as a Fibrotic Marker in the Blood

Plasma hydroxyproline (HYP) levels were increased in patients with hepatobiliary diseases (HBD) and cholangiocarcinoma (CCA) (Prakobwong et al. 2012). The data obtained of this study showed that plasma HYP may be useful as a novel predictive marker for estimating the severity score of liver fibrosis. Interestingly, HYP level in liver tissues was significantly interrelated with its plasma level and liver fibrosis scores (Prakobwong et al. 2010).

Schistosomiasis is one of the endemic diseases that affects more population and causes severe liver diseases with varying pathological changes (Crompton 1999; WHO 2010). Serum levels of HYP were reported in both *Schistosoma mansoni*-infected animal and human models with moderate splenomegaly syndrome (MSS) or hypersplenomegaly syndrome (HSS) (Manivannan et al. 2011). The results showed significant increase in the level of HYP in mice and human subjects with HSS and MSS; however, the range is very predicative in HSS samples when compared to those of MSS. This argues the importance of HYP as a valuable marker for fibrosis in HSS targets and could be used as a diagnostic marker for early detection of hepatosplenic schistosomiasis. Also, in experimentally models with hepatic fibrosis, there was a significant increase in serum HYP levels following DMN treatment (George and Chandrakasan 2000). Similarly, the elevations of serum hydroxyproline have been significantly reported in patients with alcoholic liver cirrhosis (Mata et al. 1975) and chronic liver diseases (Yamada and Hirayama 1985).

In patients with chronic hepatitis C, the severity of liver fibrosis is greatly different from one patient to another and only little percentage of patients suffers from its severe complications (Chen and Morgan 2006). Thus, treatment decisions based on histological fibrosis and necroinflammatory activity grade are critical, and other noninvasive markers to support the diagnosis and treatment interventions are needed. It was reported that liver biopsy is not perfect enough to perform a reliable diagnosis (Bedossa et al. 2003; Raja and Janjua 2008).

Hydroxyproline content was shown to be a significant marker which increased in serum and liver tissues with advancing stage of fibrosis (Lee et al. 2005).

Many research studies focused that it is important to find out new noninvasive markers for the assessment of liver histology. Many markers isolated from liver cell injury, and fibrosis was evaluated in most studies, but none of these proved to be reliable enough for clinical use (Dienstag 2002). The change in the level of hydroxyproline (Hyp) in serum, urine, and liver tissues is reported as indicator for collagen metabolism and consequently enabled with reliable important information about both pathological and biochemical changes of hepatic fibrosis (Yamada and Hirayama 1985).

Recently, hydroxyproline was estimated in the serum of chronic HCV patients. In this study, the increase in the levels of serum hydroxyproline was shown to be significantly correlated with the severity of liver fibrosis, and when hydroxyproline and other oxidative markers were applied as noninvasive diagnostic markers, significant fibrosis was predicted accurately with a range of 80–90% and cirrhosis with a range of 67–97% of HCV patients (Gabr and Alghadir 2014). According to

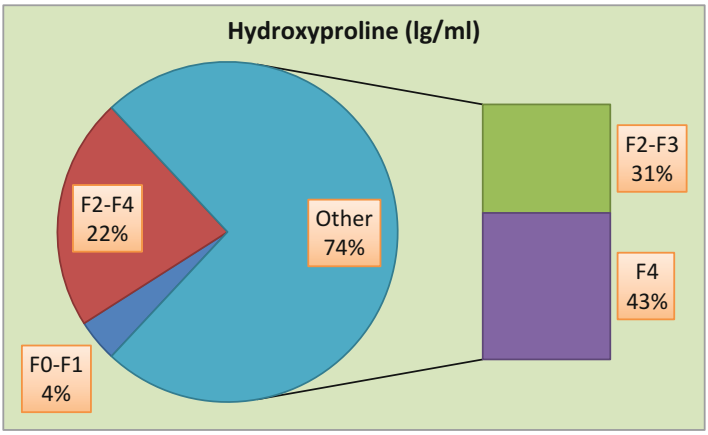

Fig. 4 Correlation between serum hydroxyproline levels estimated with colorimetric assay and severity of liver disease in hepatitis C patients. Histological analyses classified patients using the METAVIR scoring system into no fibrosis (F0), mild fibrosis (F1), moderate fibrosis (F2), severe fibrosis (F3), and cirrhosis (F4). Significant fibrosis was also defined as F2–F4. The expression of hydroxyproline was shown to be directly linked with liver fibrosis scores (Gabr and Alghadir, 2014)

histological analyses using the METAVIR scoring system, the patients in this study were classified into patients with no fibrosis (F0), mild fibrosis (F1), moderate fibrosis (F2), severe fibrosis (F3), and cirrhosis (F4). Significant fibrosis was also defined as F2–F4. The expression of hydroxyproline as a marker of liver fibrosis was shown to be significantly correlated with the severity of the disease as shown in Fig. 4. Using Hyp cutoff values, the diagnostic accuracies of significant fibrosis and cirrhosis in accordance to liver biopsy were reported in 90% and 93.3% of CHC patients with an specificity of 94%, respectively, as shown in Table 1 (Gabr and Alghadir 2014).

Hydroxyproline as a Fibrotic Marker in Liver Tissues

Liver biopsy is the most important golden method for diagnosis of liver fibrosis especially in confused liver diseases, supporting the progression of fibrosis and potency of anti-fibrotic therapy (Sherlock and Dooley 2002; Poynard et al. 1997; Shiratori et al. 2000). However, most studies showed that there was clear variability and conflicts in the diagnosis and staging of liver fibrosis during scanning of whole sections or liver biopsies (Abdi et al. 1979; Maharaj et al. 1986; Garrido and Hubscher 1996).

Quantitative measurement of hydroxyproline was reported in liver tissue samples of patients with viral hepatitis. The data obtained from this study showed that the increase in hydroxyproline content in liver tissues directly and significantly correlated with the stage of liver fibrosis (Lee et al. 2005). Also, estimation of hydroxyproline in liver tissues was used as a candidate marker to measure the anti-fibrotic

Table 1 Diagnostic accuracy of hydroxyproline content in the prediction of significant fibrosis (F2–4) and cirrhosis (F4) of 150 patients with chronic hepatitis C

Hydroxyproline (μg/ml)	Total (n)	Fibrosis		Sensitivity (95% CI)[a]	Specificity (95% CI)[a]	Positive predicative value (95% CI)[a]	Negative predictive value (95% CI)[a]
		F0–F1 (n = 60) (40%)	F0–F1 (n = 90) (60%)				
Cut off values in fibrosis							
≤1.7	85	70	15	87 (81–100)	94 (87–100)	93 (73–98)	75 (54–96)
>1.7	65	50	15				
≤4.5	95	45	25	94 (87–100)	90 (73–97)	91 (88–98)	85 (79–100)
>4.5	50	10	40				
Hydroxyproline (μg/ml)	Total (n)	Cirrhosis		Sensitivity (95% CI)[a]	Specificity (95% CI)[a]	Positive predicative value (95% CI)[a]	Negative predictive value (95% CI)[a]
		F0–F3 (n = 120;80%)	F4 (n = 30; 20%)				
Cut off values in cirrhosis							
≤6.5	60	56	4	87 (81–100)	94 (87–100)	92 (73–98)	59 (52–96)
>6.5	90	65	13				
≤8.5	70	45	15	94 (86.8–100)	75.3 (73–97)	64 (45–85)	84 (79.4–100)
>8.5	80	15	65				

[a]95% confidence interval. Cut off values of predicting fibrosis and cirrhosis patients with chronic hepatitis C were selected according to estimated area under receiver operating characteristic curves (AUROCs). The AUROCs of the hydroxyproline (Hyp) for predicting significant fibrosis and cirrhosis were 0.79 (0.70–0.85) and 0.92 (0.82–0.94), respectively (Gabr and Alghadir 2014)

effects of some chemotherapeutic drugs against liver fibrosis of rats induced by CCL_4 toxicity (El-Kholi et al. 2015).

Similarly, the quantitative measurements of hydroxyproline in liver tissue samples were greatly reported in experimental animals (mice) infected with *Schistosoma mansoni* as parasitological agent for inducing liver fibrosis. The animals were treated with some herbal plants as anti-fibrotic remedy. In this study, animals treated with herbal medicine showed significant decrease in the level of hydroxyproline content as a biochemical marker of liver fibrosis compared to control-infected group. The data concluded the potential efficacy of hydroxyproline as a pivotal candidate marker to measure the anti-fibrotic activity of herbal plants against liver fibrosis induced by *Schistosoma mansoni* (Kadry et al. 2013). The validity of hydroxyproline as a marker of liver fibrosis was also reported in liver tissues as well as serum samples of mouse and human with hepatosplenic schistosomiasis which indicates the potential use of hydroxyproline as a marker for early detection of hepatosplenic schistosomiasis (Manivannan et al. 2011).

Also, in a study performed on liver fibrosis of rats induced by CCl_4 toxicity and treated with Yi Guan Jian Decoction (YGJD), a classical traditional formula, the significant decrease in the level of hydroxyproline in YGJD treated groups proved its ability to be used as a diagnostic marker to measure the activity of these traditional medicines as anti-fibrotic agents (Gou et al. 2013).

In the same manner, the importance of hydroxyproline as a biochemical fibrotic marker was evaluated among different fibrotic markers to measure the fibrogenic activity of hepatic stellate cells (HSCs) in rat liver fibrosis induced by choline-L-amino acid-deficient diet (Hironaka et al. 2000). The data of this study showed that the increase in hydroxyproline content in liver tissues of animals treated with amino acid-deficient diet was significantly correlated with histological and other biochemical parameters such as serum PIIIP which significantly synthesized by HSCs during liver fibrosis.

Hydroxyproline and Anti-fibrotic Strategies of Herbal Medicine

Previous research reports showed that the process of liver fibrosis could be a reversible mechanism and there was a big chance to find new medicine for liver fibrosis and cirrhosis (Fallowfield et al. 2006; Fowell and Iredale 2006). However, lower effect of ordinary chemotherapeutic agents was reported on cases of chronic liver injury or severe fibrotic progression. So, medical research trials were evaluated to find out new anti-fibrotic agent of plant origin (Levy et al. 2004; Yang et al. 2008). The continued scientific experiences on traditional herbal medicine and extraction of new therapeutic compounds of plant origin gave the chance for these compounds to be used as anti-fibrotic agents against liver fibrosis (Inao et al. 2004; Li et al. 2003).

Many anti-fibrotic agents of plant origin play a significant role in reversing the severity of liver fibrosis via the antioxidant and anti-inflammatory activities of their constituents such as phenolic and flavonoid compounds which modulate the action of oxidative stress and inflammatory parameters as mediators of fibrogenesis (L'opez

et al. 2003; Rice-Evans et al. 1996). They effect on both the activity of HSCs promoting liver fibrogenesis and hydroxyproline content in liver tissues (Ahmad et al. 2009; George et al. 2006; Ahmad et al. 2011).

Some of anti-fibrotic phytochemicals are selected based on its mode of action, effect on the level of hydroxyproline content of liver tissues, and inhibition of HSCs activity or apoptosis (Table 1).

Finally, from the abovementioned scientific data, it can be concluded that the alteration of hydroxyproline levels in the liver, serum, and urine may provide unique significant information as a diagnostic marker about biochemical, severity score of hepatic fibrosis especially during diagnosis and treatment strategies using chemotherapeutic or nondrug herbal medicine.

Potential Applications to Prognosis, Other Diseases, or Conditions

Collagen is present in all human organs, and hydroxyproline constitutes the most determinant marker measuring the rate of formation and deposition of collagen. Due to its easy liberation in serum and urine with different chemical forms, it facilitates its diagnosis via immunoassay, colorimetric, and HPLC techniques. That can be used easily in diagnosis of other human diseases and disorders such as bone metabolism disorders, rheumatoid arthritis, blood vessel disorders, and lung disease (Table 2).

Summary Points

- Hydroxyproline is a remarkable indicator of any disease containing collagen variability.
- It plays a significant role in the stabilization of the integrity and the formation of extracellular matrix (ECM) fibrils produced by activated hepatic stellate cells (HSCs).
- Hydroxylation of procollagen into hydroxyproline moiety plays a pivotal role in the synthesis of ECM which preserves the structure and function of normal cells.
- Under biological and chemical agents inducing liver injury, hepatic stellate cells (HSCs) activated and more hydroxyproline increased in liver tissue and subsequently released in serum and urine.
- The excretion of hydroxyproline in serum and urine promotes the easy diagnosis of the severity of hepatic fibrosis especially in treatment interventions using herbal or non-herbal medicine strategies.
- This protein could be easily used as a noninvasive marker for diagnosis and treatment of liver fibrosis.
- The released hydroxyproline content positively correlated with the severity of liver fibrosis.
- This protein could be used as a target for diagnosis, gene therapy, and herbal medicine in patients with chronic liver complications and progression of fibrosis.

Table 2 Some phytochemicals selected as antifibrotic agents and their therapeutic actions

Phytochemical	Fibrosis model	Sample	Fibrotic marker	Mode of action	References
ShenqiNeijin powder mixture (SQNJP)	CCl_4 -toxicity	Liver	Hydroxyproline Alpha-smooth muscle actin (α-SMA) Hepatic stellate cells (HSCs)	Decrease in the level of hydroxyproline content, collagen, α-SMA Inhibiting HSCs activation and inducing apoptosis of HSCs	Xie et al. 2015
CGX-extract (mixture of 13 Korean herbs	Dimethyl nitrosamine (DMN-liver toxicity) CCl4-cell line toxicity	Liver Cell line	Hydroxyproline Antioxidant enzymes Cytokines and inflammatory modulators α-SMA Collagen and procollagen Hepatic stellate cells (HSCs)	Reduction in the level of hydroxyproline content, collagen, procollagen, α-SMA Increase in antioxidant enzyme active Down regulation of fibrogenic cytokines and inflammatory modulators Inhibiting of hepatic stellate cell activation via the downregulation of fibrogenic cytokines and antoxidant capacity	Wang et al. 2010
DiwuYanggan (DWYG)	CCl4 -toxicity	Liver Serum	Hepatic hydroxyproline content Epithelial-to mesenchymal transition (EMT) Mesenchymal-to epithelial transition (MET) Transforming growth factor β1 (TGF-β1) Bone morphogenetic protein-7 (BMP-7) E-cadherin, mesenchymal marker,vimentin	Decline in hepatic hydroxyproline content Reverse of epithelial-to-mesenchymal transition (EMT) to mesenchymal-to-epithelial transition (MET) in the fibrotic liver tissues Up-regulation expression of E-cadherin and down-regulation expression of vimentin Reduction in TGF-β1 expression Enhance in BMP-7 expression	Shen et al. 2014b

(*continued*)

Table 2 (continued)

Phytochemical	Fibrosis model	Sample	Fibrotic marker	Mode of action	References
Lithospermate B extract (*Salvia miltiorrhizae*)	Thioacetamide (TAA) -toxicity	Liver Serum HSCs culture	Hepatic hydroxyproline content α-SMA TGF-β1 and collagen α1 NF-kB transcriptional activation monocyte chemotactic protein 1 (MCP-1) H_2O_2 oxidative free radical Hepatic stellate cells (HSCs)	Decrease in hepatic hydroxyproline content, α-SMA, TGF-β1, and collagen α1 Suppression in NF-kB and (MCP-1) production Increase in antioxidant activity and suppression of oxidative stress activity Inhibition of fibrogenic responses of HSCs	Paik et al. 2011
Inchin-ko-to (TJ-135)	Choline-deficient l-amino acid-defined diet	Liver Serum HSCs culture	Hepatic hydroxyproline content Procollagen III Mitogen-activated protein (MAP) kinase Hepatic stellate cells (HSCs)	Decrease in hepatic hydroxyproline content and procollagen III Reduction in MAP-kinase expression Inhibition of fibrogenic responses of HSCs Histologically reduce the development of preneoplastic lesions	Sakaida et al. 2003
Combined mixture (*Arctiumlappa, Curcuma longa, Piperlongum, Plumbagozeylanica and Terminaliachebula*)	Ethanol -toxicity	Liver Serum Urine	Hepatic hydroxyproline content Collagen Oxidative stress free radicals (MDA) Antioxidant profile (GSH, GSSG)	Reduction in hydroxyproline content in liver tissue and urine Reduction in collagen deposition Increase in antioxidant activity and decrease in oxidative free radicals (MDA)	Kumar et al. 2011

Crude of *Reishi mushroom*, *G. lucidum* extract (GLE)	CCl4 -toxicity	Liver Serum	Hepatic hydroxyproline content Collagen Oxidative stress free radicals (MDA) Transforming growth factor β1 (TGF-β1) Methionine adenosyltransferase 1,2 (MAT1A;MAT2A)	Decrease in hepatic hydroxyproline content and collagen Reduction in MDA oxidative free radical Decrease in TGF-β1 expression as fibrosis mediator Change in the expression of MAT1A and MAT2A	Lin and Lin 2006
Astaxanthin (plant leaves)	CCl4 -toxicity	Liver Serum	Hepatic hydroxyproline content Collagen NF-*k*B and TGF-*β*1 MMP2 and TIMP1 Hepatic stellate cells (HSCs)	Decrease in hepatic hydroxyproline content and collagen Decrease in the expression of NF-*k*B and TGF-*β*1 Maintain the balance between MMP2 and TIMP1 Inhibition of hepatic stellate cells (HSCs) activation and formation of extracellular matrix (ECM)	Shen et al. 2014
Powder of cultured mycelium *Cordycepssinensis* (CMCS)	CCl4 -toxicity	Liver Serum	Hepatic hydroxyproline content α-SMA Collagen EdU, F-actin Hepatic stellate cells (HSCs	Decrease in hepatic hydroxyproline content, collagen deposition, and α-SMA level Reduction in hepatic inflammation Downregulation of EdU and F-actin levels Inhibition of fibrogenic responses of HSCs	Peng et al. 2014

Acknowledgment The project was fully financially supported by King Saud University, through Vice Deanship of Research Chairs, Rehabilitation Research Chair.

References

Abdi W, Millan JC, Mezey E. Sampling variability on percutaneous liver biopsy. Arch Intern Med. 1979;139:667–9.

Adams E, Frank L. Metabolism of proline and the hydroxyprolines. Annu Rev Biochem. 1980;49:1005–61.

Ahmad R, Ahmad S, Khan NU, Hasnain A. Operculina turpethum attenuates NDMA induced toxic liver injury and clastogenicity in rats. Chem Biol Interact. 2009;181:145–53.

Ahmad A, Fatima R, Maheshwari V, Ahmad R. Effect of N′ nitrosodimethylamine on red blood cell rheology and proteomic profiles of brain in male albino rats. Interdiscip Toxicol. 2011;4:125–31.

Bedossa P, Paradis V. Liver extracellular matrix in health and disease. J Pathol. 2003;200:504–15.

Bedossa P, Dargere D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. Hepatology. 2003;38:1449–57.

Bruck R, Genina O, Aeed H, et al. Halofuginone to prevent and treat thioacetamide-induced liver fibrosis in rats. Hepatology. 2001;33:379–86.

Chen SL, Morgan TR. The natural history of hepatitis C virus (HCV) infection. Int J Med Sci. 2006;3:47–52.

Crompton DWT. How much human helminthiasis is there in the world? J Parasitol. 1999;85:397–403.

Desmouliere A, Darby IA, Gabbiani G. Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. Lab Invest. 2003;83:1689–707.

Dienstag JL. The role of liver biopsy in chronic hepatitis C. Hepatology. 2002;36:S152–60.

Drobnik J, Dabrowski R. Melatonin suppresses the pinealectomy-induced elevation of collagen content in a wound. Cytobios. 1996;85:51–8.

El-Kholi EM, El-beltagi HM, Abdo VB, et al. Evaluation of the possible antifibrotic effect of aliskiren, valsartan, chloroquine, zafirlukast and colchicine on liver fibrosis induced by carbon tetrachloride in rats. Br J Med Health Res. 2015;2(7):41–54.

Elsisi AE, Elfert AA, Elsayad M, Zakaria S. A randomized controlled study of the effect of AT1 antagonist on fibrosis markers in HCV Egyptian patients. J GHR. 2012;1(9):217–22.

Fallowfield JA, Kendal TJ, Iredale JP. Reversal of fibrosis: no longer a pipe dream? Clin Liver Dis. 2006;10:481–97.

Fowell AJ, Iredale JP. Emerging therapies for liver fibrosis. Dig Dis. 2006;24:174–83.

Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem. 2000;275:2247–50.

Gabr SA, Alghadir AH. Prediction of fibrosis in hepatitis C patients: assessment using hydroxyproline and oxidative stress biomarkers. Indian J Virol. 2014;25:91–100.

Garrido MC, Hubscher SG. Accuracy of staging in primary biliary cirrhosis. J Clin Pathol. 1996;49:556–9.

Gasser AB, Depierre D, Courvoisier B. Total urinary and free serum hydroxyproline in metastatic bone disease. Br J Cancer. 1979;39:280–3.

George J, Chandrakasan G. Biochemical abnormalities during the progression of hepatic fibrosis induced by dimethylnitrosamine. Clin Biochem. 2000;33(7):563–70.

George J, Suguna L, Jayalakshmi R, Chandrakasan G. Efficacy of silymarin and curcumin on dimethylnitrosamine induced liver fibrosis in rats. Biomedicine. 2006;26:18–26.

Gordon MK, Hahn RA. Collagens. Cell Tissue Res. 2010;339:247–57.

Gorres KL, Raines RT. Prolyl-4 hydroxylase. Crit Rev Biochem Mol Biol. 2010;45(2):106–24. doi: 10.3109/10409231003627991.

Gou X, Tao Q, Feng Q, et al. Urine metabolic profile changes of CCl4-liver fibrosis in rats and intervention effects of Yi Guan Jian Decoction using metabonomic approach. BMC Complement Altern Med. 2013;3(13):123. doi:10.1186/1472-6882-13-123.

Hironaka K, Sakaida I, Uchida K, Okita K. Correlation between stellate cell activation and serum fibrosis markers in choline-deficient L-amino acid-defined diet-induced rat liver fibrosis. Dig Dis Sci. 2000;45(10):1935–43.

Hu CA, Khalil S, Zhaorigetu S, et al. Human Δ pyrroline5carboxylate synthase: function and regulation. Amino Acids. 2008;35:665–72.

Inao M, Mochida S, Matsui A, et al. Japanese herbal medicine Inchin-ko-to as a therapeutic drug for liver fibrosis. J Hepatol. 2004;41:584–91.

Jiang JX, Torok NJ. Liver injury and the activation of the hepatic myofibroblasts. Curr Pathobiol Rep. 2013;1(3):215–23.

Kadry SM, Mohamed AM, Farra EM, Fayed DM. Influence of some micronutrients and Citharexylum quadrangular extract against liver fibrosis in *Schistosoma mansoni* infected mice. Glob J Pharm Pharmacol. 2013;1(1):051–61.

Kaul S, Sharma SS, Mehta IK. Free radical scavenging potential of Lproline: evidence from in vitro assays. Amino Acids. 2008;34:315–20.

Kisseleva T, Brenner DA. Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. J Gastroenterol Hepatol. 2007;22(1):S73–8.

Knight J, Holmes RP. Mitochondrial hydroxyproline metabolism: implications for primary hyperoxaluria. Am J Nephrol. 2005;25:171–5.

Krane SM. The importance of proline residues in the structure, stability and susceptibility to proteolytic degradation of collagens. Amino Acids. 2008;35:703–10.

Kumar S, Kumari P, Devi S. Antioxidative role of selected herbs against ethanol induced liver injury in rats. J Appl Nat Sci. 2011;3(2):242–6.

L'opez M, Mart'ınez F, Del Valle C, Ferrit M, Luque R. Study of phenolic compounds as natural antioxidants by a fluorescence method. Talanta. 2003;60(2–3):609–16.

Laitinen O, Nikkila EA, Kivirikko KI. Hydroxyproline in the serum and urine. Acta Med Scand. 1966;179:275–84.

Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med. 2001;345:41–52.

Laurent GJ, McAnulty RJ. Protein metabolism during bleomycininduced pulmonary fibrosis in rabbits: in vivo evidence for collagen accumulation because of increased synthesis and decreased degradation of the newly synthesized collagen. Am Rev Respir Dis. 1983;128: 82–8.

Lee HS, Shun CT, Chiou LL, Chen CH, Huang GT, Sheu JC. Hydroxyproline content of needle biopsies as an objective measure of liver fibrosis: emphasis on sampling variability. J Gastroenterol Hepatol. 2005;20:1109–14.

Levy C, Seeff LD, Lindor KD. Use of herbal supplements for chronic liver disease. Clin Gastroenterol Hepatol. 2004;2:947–56.

Li D, Friedman SL. Liver fibrogenesis and the role of hepatic stellate cells: new insights and prospects for therapy. J Gastroenterol Hepatol. 1999;14:618–33.

Li C, Luo J, Li L, et al. The collagenolytic effects of the traditional Chinese medicine preparation, Han-Dan-Gan-Le, contribute to reversal of chemical-induced liver fibrosis in rats. Life Sci. 2003;72:1563–71.

Lin W-C, Lin W-L. Ameliorative effect of *Ganoderma lucidum* on carbon tetrachloride-induced liver fibrosis in rats. World J Gastroenterol. 2006;12(2):265–70.

Lindblad WJ, Diegelmann RF. Quantitation of hydroxyproline isomers in acid hydrolysates by high-performance liquid chromatography. Anal Biochem. 1984;138:390–5.

Lotersztajn S, Julin B, Teixeira-Clerc F, Grenard P, Mallat A. Hepatic fibrosis: molecular mechanisms and drug targets. Annu Rev Pharmacol Toxicol. 2005;45:605–28.

Maharaj B, Maharaj RJ, Leary WP, et al. Sampling variability and its influence on the diagnostic yield of percutaneous needle biopsy of the liver. Lancet. 1986;1(8480):523–25.
Manivannan B, Rawson P, Jordan TW, et al. Identification of cytokeratin 18 as a biomarker of mouse and human hepatosplenic schistosomiasis. Infect Immun. 2011;79(5):2051–8.
Mata JM, Kershenobich D, Villarreal E, Rojkind M. Serum free proline and free hydroxyproline in patients with chronic liver disease. Gastroenterology. 1975;68:1265–9.
McAnulty RJ, Laurent GJ. Collagen synthesis and degradation in vivo. Evidence for rapid rates of collagen turnover with extensive degradation of newly synthesized collagen in tissues of the adult rat. Coll Relat Res. 1987;7:93–104.
McAnulty RJ, Staple LH, Guerreiro D, Laurent GJ. Extensive changes in collagen synthesis and degradation during compensatory lung growth. Am J Physiol Cell Physiol. 1988;255:C754–9.
McAnulty RJ, Moores SR, Talbot RJ, Bishop JE, Mays PK, Laurent GJ. Long-term changes in mouse lung following inhalation of a fibrosis-inducing dose of $239PuO_2$: changes in collagen synthesis and degradation rates. Int J Radiat Biol. 1991;59:229–38.
McAnulty RJ, Chambers RC, Laurent GJ. Regulation of fibroblast procollagen production: transforming growth factor-β1 induces prostaglandin E2 but not procollagen synthesis via a pertussis toxin-sensitive G-protein. Biochem J. 1995;307:63–8.
Murawaki Y, Yamada S, Koda M, Hirayama C. Collagenase and collagenolytic cathepsin in normal and fibrotic rat liver. J Biochem. 1990;108:241–4.
Need AG. Bone resorption markers in vitamin D insufficiency. Clin Chim Acta. 2006;368:48–52.
Nelson DL, Cox MM, editors. Lehninger's principles of biochemistry. 4th ed. New York: W. H. Freeman and Company; 2005.
Nobbs BT, Walke AW, Davies TJ. A simplified method for the estimation of urinary total hydroxyproline. Clin Chim Acta. 1975;64:219–21.
Paik YH, Yoon YJ, Lee HC, et al. Antifibrotic effects of magnesium lithospermate B on hepatic stellate cells and thioacetamide-induced cirrhotic rats. Exp Mol Med. 2011;43(6):341–9.
Peng Y, Tao Y, Wang Q, et al. Ergosterol is the active compound of cultured mycelium cordyceps sinensis on antiliver fibrosis. Evid Based Complement Alternat Med. 2014;537234:1–12. doi: 10.1155/2014/537234.
Peterkofsky B, Prockop DJ. A method for the simultaneous measurement of radioactivity of proline-C14 and hydroxyproline-C14 in biological materials. Anal Biochem. 1962;4:400–6.
Phang JM, Hu CA, Valle D. Disorders of proline and hydroxyproline metabolism. In: Scriver CR, Beaudet AL, Sly WS, Vallee D, Childs B, Kinzler KW, Vogelstein B, editors. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill; 2001. p. 1821–38.
Pihlajaniemi T, Myllyla R, Kivirikko KI. Prolyl 4-hydroxylase and its role in collagen synthesis. J Hepatol. 1991;13 Suppl 3:S2–7.
Ping L, Cheng L, Lie-Ming X. Effects of Fuzheng Huayu 319 recipe on liver fibrosis in chronic hepatitis B. WJG. 1998;4(4):348–53.
Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet. 1997;349:825–32.
Prakobwong S, Yongvanit P, Hiraku Y, et al. Involvement of MMP-9 in peribiliary fibrosis and cholangiocarcinogenesis via Rac1-dependent DNA damage in a hamster model. Int J Cancer. 2010;127:2576–87.
Prakobwong S, Charoensuk L, Hiraku Y, et al. Plasma hydroxyproline, MMP-7 and collagen I as novel predictive risk markers of hepatobiliary disease-associated cholangiocarcinoma. Int J Cancer. 2012;131:E416–24.
Prockop DJ, Udenfriend S. A specific method for the analysis of hydroxyproline in tissues and urine. Anal Biochem. 1960;1:228–39.
Prockop DJ, Udenfriend S, Lindstedt S. A simple technique for measuring the specific activity of labelled hydroxyproline in biological materials. J Biol Chem. 1961;236:1395–8.
Raja NS, Janjua KA. Epidemiology of hepatitis C virus infection in Pakistan. J Microbiol Immunol Infect. 2008;41:4–8.

Rice-Evans CA, Miller NJ, Paganga G. Structure antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med. 1996;20(7):933–56.
Sakaida I, Tsuchiya M, Kawaguchi K, Kimura T, Terai S, Okita K. Herbal medicine Inchin-ko-to (TJ-135) prevents liver fibrosis and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet. J Hepatol. 2003;38(6):762–9.
Shen M, Chen K, Lu J, et al. Protective effect of astaxanthin on liver fibrosis through modulation of TGF-β1 expression and autophagy. Mediat Inflamm. 2014;954502:1–14. doi: 10.1155/2014/954502.
Shen X, Cheng S, Peng Y, Song H, Li H. Attenuation of early liver fibrosis by herbal compound "Diwu Yanggan" through modulating the balance between epithelial-to-mesenchymal transition and mesenchymal-to-epithelial transition. BMC Complement Altern Med. 2014b;14:418. doi:10.1186/1472-6882-14-418.
Sherlock S, Dooley J. Diseases of the liver and biliary system. 11th ed. Oxford: Blackwell Science; 2002.
Shiratori Y, Imazeki F, Moriyama M, et al. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. Ann Intern Med. 2000;132:517–24.
Spira G, Mawasi N, Paizi M, et al. Halofuginone: a collagen type I inhibitor improves liver regeneration in cirrhotic rats. J Hepatol. 2002;37(3):331–9.
Stalnikowitz DK, Weissbrod AB. Liver fibrosis and inflammation. A review. Ann Hepatol. 2003;2(4):159–63.
Tacke F, Weiskirchen R. Update on hepatic stellate cells: pathogenic role in liver fibrosis and novel isolation techniques. Expert Rev Gastroenterol Hepatol. 2012;6(1):67–80.
Tsuchiya H, Bates CJ. Vitamin C and copper interactions in guinea pigs and a study of collagen cross links. Br J Nutr. 1997;77:315–25.
Wang JH, Shin JW, Son JY, Cho JH, Son CG. Antifibrotic effects of CGX, a traditional herbal formula, and its mechanisms in rats. J Ethnopharmacol. 2010;127(2):534–42.
White ES, Lazar MH, Thannickal VJ. Pathogenetic mechanisms in usual interstitial pneumonia/idiopathic pulmonary fibrosis. J Pathol. 2003;201:343–54.
WHO. Schistosomiasis. Geneva: WHO; 2010. http://www.who.int/mediacentre/factsheets/fs115/en/index.html.2010
Wu G, Bazer FW, Burghardt RC, et al. Proline and hydroxyproline metabolism: implications for animal and human nutrition. Amino Acids. 2010;40(4):1053–63.
Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol. 2008;214(2):199–210.
Xie H, Hou W, Yang Y, Yu Y, Wang F, Mao J. Effects of Shenqi Neijin powder on activation and apoptosis of hepatic stellate cells in rats with hepatic fibrosis. Int J Clin Exp Med. 2015;8(2):2226–32.
Yamada S, Hirayama C. Clinical significance of serum hydroxyproline containing peptides with special reference to hyproprotein. Eur J Clin Invest. 1985;13:129–33.
Yang Y, Yang S, Chen M, Zhang X, Zou Y, Zhang X. Compound Astragalus and Salvia miltiorrhiza extract exerts anti-fibrosis by mediating TGFbeta/Smad signaling in myofibroblasts. J Ethnopharmacol. 2008;118:264–70.

Fibrinogen α-Chain as a Serum Marker of Liver Disease

23

Santiago Marfà and Wladimiro Jimenez

Contents

S. Marfà (✉)
Biochemistry and Molecular Genetics Service, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain
e-mail: marfa@clinic.ub.es; santimarfa@gmail.com

W. Jimenez
Biochemistry and Molecular Genetics Service, Biochemistry and Molecular Genetics Department, Hospital Clinic of Barcelona, Barcelona, Spain

Department of Biomedicine, University of Barcelona, Barcelona, Spain

Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Barcelona, Spain
e-mail: wjimenez@clinic.ub.es; wjimenez@ub.edu

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_7

Abstract

Liver fibrosis is the hepatic response to an insult characterized by an accumulation of extracellular matrix proteins. If the underlying cause is not treated or eliminated, the disease can progress and may lead to several clinical complications including hepatocellular carcinoma or even death. Thus, detection, staging, and follow-up of liver fibrosis are the main issues in the prognosis and treatment of patients with chronic liver disease. In recent years, new advances in mass spectrometry-based proteomics technology and protein fractionation techniques have improved protein identification as well as protein quantification in many different samples and diseases including liver fibrosis. In particular, the fibrinogen α chain and more specifically the serum levels of the 5.9 kDa fragment of fibrinogen α C-chain have shown to be altered in several hepatic etiologies. In fact, these results have been reproduced by different laboratories, and recently a marked downregulation of this protein fragment has also been described in the initial stages of liver fibrosis. In this chapter, we have described the potential role of fibrinogen α chain and particularly the 5.9 kDa fragment of fibrinogen α C-chain as a circulating marker of liver fibrosis.

Keywords

Biomarker • Liver • Early detection • Fibrinogen • Fibrinogen alpha chain • Fibrosis • Mass spectrometry • Proteomics

List of Abbreviations

AH	Autoimmune hepatitis
ALD	Alcoholic liver disease
CDT	Carbohydrate-deficient transferring
ECM	Extracellular matrix
ELF	Enhanced liver fibrosis
ESI	Electrospray ionization
GGT	Gamma-glutamyltransferase
HA	Hyaluronic acid
HBV	Hepatitis B virus
HCV	Hepatitis C virus
LT	Liver transplantation
m/z ratio	Mass-to-charge ratio
MALDI-TOF/TOF MS	Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry
MS	Mass spectrometry
NASH	Nonalcoholic steatohepatitis
PIIINP	Amino-terminal peptide of type III procollagen
SELDI-TOF-MS	Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry
SID-MS	Stable-isotope dilution mass spectrometry
TGF-β	Transforming growth factor beta

TIMP-1	Tissue inhibitor of matrix metalloproteinase-1
TOF	Time of flight

Key Facts of Liver Function and Structure

- The liver is the largest organ in the body and is located in the upper-right region of the abdominal cavity.
- It is comprised of four different lobes.
- It is the only organ that has a dual blood supply: the hepatic artery and the portal vein.
- While the hepatic artery provides the oxygen necessary to achieve the different vital functions, the portal vein, which comes from the intestines, is detoxified in the organ. Both blood flows converge and form the hepatic vein which finally drains into the inferior vena cava.
- Several cell types compose this organ, and they can be classified as parenchymal and non-parenchymal cells.
- Hepatic stellate cells (HSC), Kupffer cells, and hepatocytes are the most relevant cell types. The latter, however, are the principal cell types in the liver and occupy around 80% of its volume.
- The liver, but generally hepatocytes, executes many different functions including protein synthesis, bile synthesis and its secretion, toxin metabolism, etc.

Key Facts of Mass Spectrometry (MS)

- It was invented by J. J. Thomson.
- Its function is to separate, identify, and even quantify molecules based on their mass-to-charge ratio (m/z). However, molecules must be firstly converted into gaseous ions.
- A mass spectrometer consists of three basic elements:
  - Ion source: this is where the sample is ionized. Examples of this component are electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), and surface-enhanced laser desorption/ionization (SELDI).
  - Mass analyzer: this is where molecules are separated according to their mass-to-charge ratio (m/z). Examples of this component are time of flight (TOF) and quadrupole ion trap, among others.
  - Ion detector: this is used to detect and record the relative abundance of each charged molecule.

Definitions of Words and Terms

Alternative splicing	This is a biological process by which multiple mRNAs (and subsequently proteins) are generated from the same

	gene. Basically, DNA is transcribed into a pre-mRNA, which contains both introns and exons. Subsequently, mature mRNA is formed by the total or partial retention of exons, creating a diverse panel of mRNA from a single pre-mRNA. This phenomenon enhances proteome diversity and the functional capacity of each gene.
Coagulation	This is a physiological process by which fibrinogen is converted to fibrin by the action of thrombin. This cleavage leads to the appearance of a fibrin clot which turns liquid blood into a thickened mass.
Fibrinolysis	This is the mechanism by which fibrin clots are broken down by the action of plasmin. Its physiological function is to prevent the growth of blood clots and reduce their potential side effects.
HA	Hyaluronic acid is one of the components of extracellular matrix (ECM). Its serum levels have proven to have a good correlation with the severity of liver disease. In fact, it is an excellent tool to identify late stages of liver fibrogenesis. However, it is not as useful for the detection of early fibrotic stages.
HCV	Hepatitis C virus is one of the main causes of liver fibrosis and cirrhosis worldwide and the main indication for liver transplantation. However, recent advances in drug development have allowed the obtaining of better and highly effective drugs that will predictably reduce all the clinical complications caused by this virus.
Liver biopsy	Until recently, liver biopsy has been the gold standard method to stage liver fibrosis. This invasive procedure involves the removal of a small sample of liver tissue to be further histologically examined to determine the underlying cause and the severity of the liver disease.
m/z ratio	The mass-to-charge ratio is the relation between the mass (m) of a molecule and its charge number (z). In this chapter, the molecules analyzed by MS are proteins. In addition, since many of the ions carry a charge of +1, the m/z ratio is often considered to be the molecular weight of the compound.
MALDI-TOF/TOF MS	This is a proteomic approach that allows the analysis, identification, and even quantification of several biomolecules including proteins. It can be used for biomarker discovery.
NASH	Nonalcoholic steatohepatitis is one of the most advanced consequences of nonalcoholic fatty liver diseases (NAFLD). Subsequently, NASH can lead to cirrhosis, liver failure, or even HCC. In terms of prevalence, this phenomenon has

	increased in Western societies during the last decades due to sedentary habits, unhealthy diet, and the metabolic syndrome. Basically, it is characterized by fat accumulation in the liver which causes inflammation and damage.
Posttranslational modifications	These are biological mechanisms that enhance proteome diversity. In addition, they play a pivotal role in several cellular processes. Posttranslational modifications (PTM) include all the modifications generated during or after protein synthesis. Phosphorylation, methylation, and proteolysis are just some examples of the wide variety of these modifications.
Scheuer classification	This is one of the most commonly used classifications for the assessment of chronic hepatitis in liver biopsy specimens. Basically, liver fibrosis can be staged from zero to four. No fibrosis corresponds to fibrosis stage 0 (F0); minimal portal fibrosis would be staged as F1, periportal fibrosis (F2), fibrosis beyond the portal tract making septums (F3), and, finally, cirrhosis (F4).
SELDI-TOF-MS	It is a high-throughput proteomic approach similar to MALDI-TOF, which is also used for biomarker discovery. However, it incorporates solid-phase chromatography that reduces the complexity of the biological sample protein and allows better selectivity of the proteins of interest.
TGF-β	Transforming growth factor beta is mainly produced by Kupffer cells and is the most important cytokine that promotes wound healing and repair. TGF-β induces apoptosis of hepatocytes and activates HSC.
TIMP-1	Tissue inhibitor of metalloproteinase 1 is produced by several types of hepatic cells and inhibits the degradation of newly formed collagen fibrils by MMPs. It is also highly upregulated during fibrogenic processes.

Introduction

Liver fibrosis is a dynamic and reversible wound-healing process characterized by the accumulation of extracellular matrix proteins (ECM) in response to chronic injury (Bataller and Brenner 2005). Despite the remarkable regenerative capacity of the liver, if the underlying cause is not treated and eliminated, chronic liver injury can lead to cirrhosis, hepatocellular carcinoma, liver failure, or even death (Bataller and Brenner 2005; Lee and Friedman 2011; Forner et al. 2012; Schuppan and Kim 2013; Tsochatzis et al. 2014). Many different causes can contribute to liver fibrosis. However, the main etiologies in developed countries

are alcoholic liver disease (ALD), cholestasis, chronic hepatitis B virus (HBV) and C virus (HCV) infections, and, recently, nonalcoholic steatohepatitis (NASH) (Schuppan and Kim 2013; Friedman 2010). Knowledge of the extent of hepatic fibrosis is critical to define and establish its prognosis and progression as well as make treatment decisions. However, in clinical practice, early detection of liver fibrosis is, in many cases, relatively complex since the detection and clinical symptoms of hepatic decompensation can occur within weeks, months, or even years after the onset of injury (Hernandez-Gea and Friedman 2011). In fact, some studies have pointed out that about 40% of patients with liver disease are asymptomatic for 10–15 years before any clinical complication appears (Heidelbaugh and Bruderly 2006). Thus, prompt detection of hepatic fibrogenesis is of major importance in the prognosis and treatment of patients with chronic liver disease.

At present, liver biopsy remains the gold standard method to stage liver fibrosis (Fernandez-Varo and Jimenez 2011). Nevertheless, this procedure must be performed by a trained physician due to its invasiveness. In addition, it may involve several clinical complications including pain, hemorrhage, potential morbidity, or even death. Furthermore, the small amount of specimen obtained in the liver biopsy might not represent the fibrosis stage of the whole organ due to the heterogeneity of fibrosis. Moreover, interobserver variability should also be taken into account, and liver biopsy does not allow fibrosis progression/reversion follow-up (Castera and Pinzani 2010; Bedossa et al. 2003; Fernandez-Varo and Jimenez 2011). For these reasons, many efforts have been made during the last decade to find new noninvasive procedures as alternatives to liver biopsy. These efforts have essentially been focused on developing imaging techniques and identifying new circulating biomarkers (Carrión et al. 2010; Castera et al. 2008; Crespo et al. 2012). Despite both options having several important advantages including their noninvasiveness and easy sequential follow-up of the disease, the latter allows better accessibility (Alrawashdeh and Crnogorac-Jurcevic 2011).

Ideally, a marker of liver fibrosis should be specific and highly sensitive when discriminating the different stages of liver fibrosis of any etiology. It should also be easy to measure and economical as well as reproducible. In addition, it should not vary with inflammation or with any metabolic disorder and should be useful to monitor fibrosis progression/regression (Baranova et al. 2011). Finally, a biomarker should also be able to discriminate the underlying causes of liver disease. Nonetheless, there is currently no marker that fulfills all these criteria (Watkins 2009). However, the advances made in this field have allowed the identification of new potential and very promising biological markers. Additionally, the combination of several serum markers appears to be another interesting strategy in discriminating different stages of liver fibrosis (Sebastiani et al. 2006; Castera 2012; Crespo et al. 2012). In fact, one of the most well-known algorithms is enhanced liver fibrosis (ELF), which combines serum levels of three different direct biological markers: HA, PIIINP, and TIMP-1. This algorithm was developed in an international multicenter study including a cohort of 1,021 patients

with different hepatic etiologies (Rosenberg et al. 2004). Age was initially included in the algorithm but was finally excluded on validation of the ELF tool. In fact, ELF is highly accurate for detecting patients with significant fibrosis and cirrhosis (Martinez et al. 2011; Crespo et al. 2012). However, several studies have reported lower diagnostic capacity in early and mild stages of hepatic fibrosis in which the values obtained from fibrotic patients are similar to those of healthy subjects (Martinez et al. 2011; Lichtinghagen et al. 2013). In fact, this is a common trend in many other noninvasive markers and algorithms. Thus, discriminating early stages of liver fibrosis has remained a challenge in this disease.

In view of the fact that hepatocytes, the predominant cell type in the liver, are responsible for the synthesis of most intrahepatic and extrahepatic proteins including plasma coagulation proteins such as prothrombin or fibrinogen (Tennent et al. 2007), it is feasible to think that even subtle changes in liver architecture can incur abnormalities in protein synthesis and be further detected in the bloodstream. Thus, circulating proteins have been one of the most studied areas of research. Recent advances in mass spectrometry-based proteomics technology and protein fractionation techniques have improved protein identification as well as protein quantification in many different samples and diseases (Aebersold and Mann 2003; Camerini and Mauri 2015). In particular, thanks to these technical advances, not only changes in many abundant and non-abundant proteins can now be detected but also in protein fragments released into the circulation (Hortin 2006). In this context, some studies have demonstrated alterations in circulating serum fibrinogen fragments.

Fibrinogen Structure

Human fibrinogen is one of the most abundant proteins in blood and plays a pivotal role in the coagulation cascade (Mosesson et al. 2001; Davalos and Akassoglou 2012). Structurally, this 340 kDa acute phase glycoprotein synthesized in the liver is comprised of two symmetric sets of three different chains known as Aα, Bβ, and γ. The predominant Aα chain is the longest polypeptide chain, containing 610 residues and weighs 63.5 kDa. The Bβ (56 kDa) and γ (48 kDa) chains consist of 461 and 411 amino acids, respectively. However, all the fibrinogen chains are very heterogeneous as a result of alternative splicing, posttranslational modification, and even proteolytic degradation (Herrick et al. 1999). The N-terminus of each chain is bound by disulfide knots, forming its central E domain. In this region, the Aα and Bβ chains present a small peptide sequence known as fibrinopeptide A and B, respectively, which are crucial for preventing the conversion of fibrinogen to fibrin. At the opposite end, the C-terminus fragments of the Bβ and γ chains together with a portion of the Aα chain constitute both outer D domains. The end peptide sequence of the Aα chain is called the αC domain and forms "free-swimming appendages" (Doolittle 1973) which can interact not only with each other but with the central

region of the molecule or even with other αC domains (intermolecular interactions) in fibrin polymerization. In addition, this region is susceptible to being cleaved from fibrinogen by several proteases including plasmin and being released into the systemic circulation where it is further degraded (Cesarman-Maus and Hajjar 2005). However, some fragments from this region can be detected before their dissolution and can be used as potential biomarkers of many diseases including liver fibrosis.

Coagulation and Fibrinolysis

Under physiological conditions, both coagulation and fibrinolysis mechanisms are precisely regulated by many different elements (Cesarman-Maus and Hajjar 2005). Due to different stimuli, fibrinogen is converted to fibrin by thrombin. Afterward, the fibrin clot is broken down in order to prevent excessive clot formation and subsequently blood vessel blockage. Several factors are involved in this complex process, although plasmin is basically the enzyme that lyses the fibrin mesh at different locations, generating fibrinogen fragments of different sizes (Cesarman-Maus and Hajjar 2005). The resulting fragments are released into the circulation and are subsequently degraded by other proteases. However, this process is accelerated during liver injury (Takahashi et al. 1990). Furthermore, it has also been well established that these breakdown fragments can exert direct effects on tissue repair (Herrick et al. 1999). Therefore, it is possible that the soluble fragments released into the bloodstream or even the molecules related to coagulation or fibrinolysis processes could be differentially expressed in liver diseases and be used as biomarkers of hepatic fibrosis. Nevertheless, it seems that depending on the etiology, the mechanisms of degradation and even the cleavage sites could be different. In fact, some studies have reported that conformational changes occur in fibrinogen molecule after moderate alcohol consumption (Gorinstein et al. 2003). It is also well known that there is a lower concentration of serum fibrinogen levels in patients with moderate alcohol consumption (Sierksma et al. 2002). Additional investigations have suggested that moderate consumption entails no changes in fibrinolysis (van Golde et al. 2002), despite other studies describing the presence of clear changes in this mechanism (Dimmitt et al. 1998).

Fibrinogen as a Marker of Liver Cirrhosis

It is well established that serum fibrinogen levels remain unaltered in patients with stable liver diseases. However, this behavior differs in patients with more advanced hepatic fibrosis (de Maat et al. 1995). In fact, several studies have reported that fibrinogen levels are usually diminished in these patients, even though some cirrhotic patients may present fibrinogen serum concentrations within the normal range

(Pluta et al. 2010). It is of note that Child-Pugh C cirrhotic patients show the lowest serum levels, probably due to an overall reduction of protein synthesis in this stage. Furthermore, dysfibrinogenemia is also often observed in patients with acute liver failure (Mistry and Jain 2011). Therefore, serum fibrinogen alterations seem to mainly be found in advanced liver disease, and only in very specific cases such as acute liver failure are early alterations observed.

Early stages of liver fibrosis are associated with a clear inflammatory process. In this context, the analysis of fragmentome (also called peptidome) may play a pivotal role for its detection. The fragmentome approach is the analysis of the entire set of tiny peptide components most of which are the fruit of the degradation of the abundant proteins in serum or plasma (Hortin 2006). In fact, it has been reported that fragmentome modifications may be better indicators of inflammation and even organ injury than intact proteins. With regard to fibrinogen, several molecular fragments of the α, β, and γ chains are cleaved and released into the bloodstream due to several stimuli. A more in-depth discussion of β and γ chain fragments is outside the scope of this chapter. However, α chain fragments are particularly interesting due to their rapid alteration in early stages in liver fibrosis (Marfà et al. 2014). Indeed, recent studies using MS technology have identified altered fragments of the fibrinogen α chain with a 5.9 kDa C-terminal fragment of the fibrinogen α chain being one the most frequently described.

5.9 kDa Fibrinogen Alpha C-Chain Fragment as a Marker of ALD

At present, determination of blood ethanol levels is performed for recent alcohol intake. On the other hand, there are several biochemical markers for the diagnosis of ALD. Among them, carbohydrate-deficient transferrin (CDT) and gamma-glutamyltransferase (GGT) are the most commonly used (Anton et al. 2002). However, in some heavy drinkers, no alterations are observed in blood, with GGT serum concentrations being within the standard range (Matsuda et al. 1993).

The first study to identify and describe a clear alteration in the expression of this fibrinogen fragment in ALD was in 2004 (Nomura et al. 2004). In this study, the fibrinogen α C-chain fragment was detected by SELDI-TOF-MS. Serum levels of this circulating fragment were found to be downregulated at admission and significantly increased after 3 months of abstinence from alcohol (Fig. 1). Thus, this investigation demonstrated that this peptide allowed the monitoring of potential relapses as well as alcohol abstinence. In addition, it is of note that this circulating biomarker was able to detect all the heavy drinkers, including patients with normal GGT serum levels which were unaltered in 25% of cases, thereby establishing its diagnostic value in ALD (Sogawa et al. 2007) and highlighting its promising role as a potential biomarker of alcohol intake. However, the number of patients included in the study was low and the vast majority were men (94%). Moreover, all had consumed more than 100 g of alcohol per day for a minimum of 10 years, indicating that all of these subjects most likely had

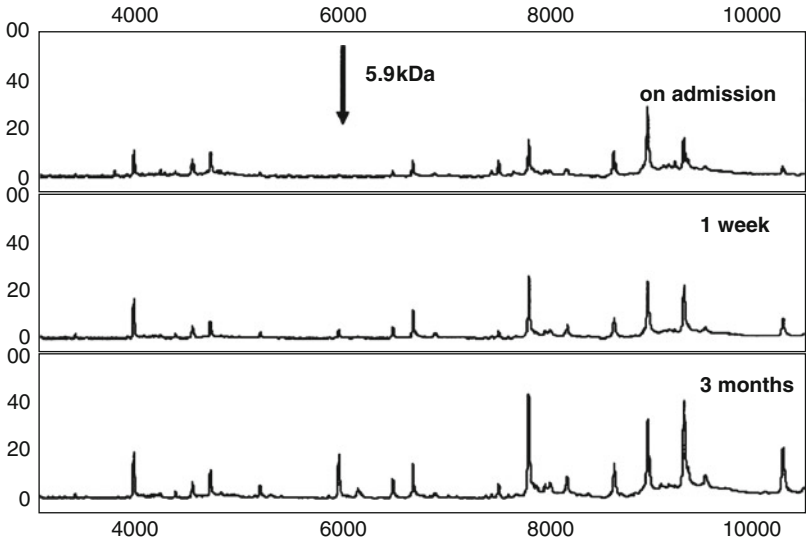

Fig. 1 Differential proteomic profile between ALD patients at admission and during alcohol abstinence. Representative portion of the SELDI-TOF-MS spectra comprised between 4,000 and 10,000 m/z of serum samples obtained from ALD patients at admission and at 1 week and 3 months of abstinence from alcohol. The upregulation of the 5.9 kDa fibrinogen α C-chain fragment is clearly associated with the time of alcohol abstinence (Figure from Nomura et al. (2004) with permission from the Publisher John Wiley and Sons, Inc)

advanced rather than early liver fibrosis. Nonetheless, these results were validated a few years later by the same group using the MALDI-TOF/TOF MS approach (Sogawa et al. 2009). In the same study, three more altered peptides were also detected by MALDI-TOF/TOF MS in serum samples from heavy drinkers and were identified as fragments of fibrinopeptide A (m/z 1466), phosphorylated fibrinopeptide A (m/z 1616), and the fibrinogen α C-chain (m/z 2660).

Later, Sogawa et al. (2011) performed another study in a group of healthy subjects and patients with different liver diseases, including ALD. Basically, the investigation was focused on the quantitative determination of the 5.9 kDa fragment of fibrinogen α chain by the use of the MALDI-TOF-MS method with a stable-isotope dilution mass spectrometry (SID-MS). This widely used MS approach, which allows the discovery and the quantitative determination of potential circulating disease biomarkers, was able to determine the 5.9 kDa α fibrinogen fragment more accurately than ordinary MS. Ultimately, Noda et al. (2011) developed a quantitative enzyme-linked immunosorbent assay (ELISA) which was able to perform a more accessible and affordable quantification analysis to determine 5.9 kDa fragment levels in serum samples from patients with ALD and healthy subjects. However, taking into account the complexity of working with specific peptide fragments and not the whole protein, this technique was characterized and validated using the SID-MS approach, showing a good correlation with the ELISA outcomes (Fig. 2).

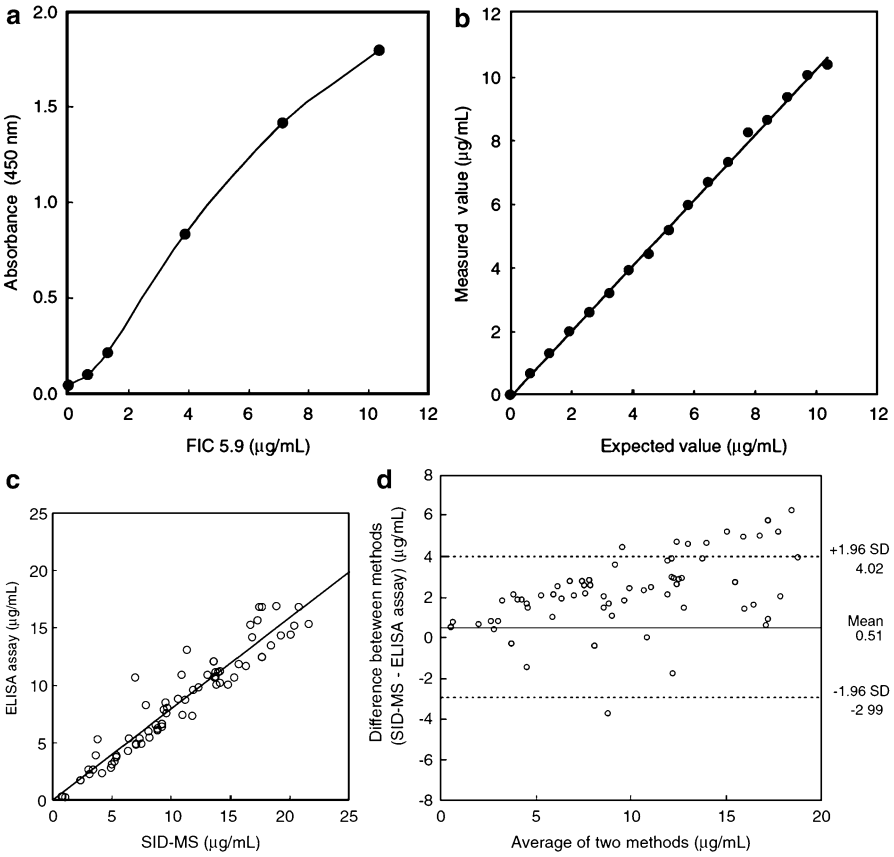

Fig. 2 Characterization of the ELISA assay and comparison to the SID-MS method. (**a**) Standard curve between the 5.9 kDa fibrinogen α C-chain fragment concentration (μg/ml) and colorimetric measures (450 nm). (**b**) ELISA validation test of linearity ($r^2 = 0.998$). (**c**) Correlation of the 5.9 kDa fibrinogen α C-chain fragment concentration with SID-MS results. (**d**) Examination of the agreement between SID-MS and ELISA procedures (Bland-Altman plot). Results are given as mean (*solid line*) ± 1.96 SD (*dotted lines*) (Modified figure from Noda et al. (2011) with permission from the Publisher John Wiley and Sons, Inc)

Early Serum 5.9 kDA Fibrinogen Alpha C-Chain Fragment Alterations in HCV Patients

In 2011, Sogawa et al. demonstrated for the first time a subtle downregulation in the 5.9 kDa fragment of fibrinogen α C-chain in serum samples from HCV patients compared to a control group. These differences were increased when the samples were analyzed by the SID-MS approach. However, this observation was very preliminary as only eight HCV patients were included in this investigation. Afterward, Sogawa et al. (2013) analyzed 88 serum samples from HCV patients and

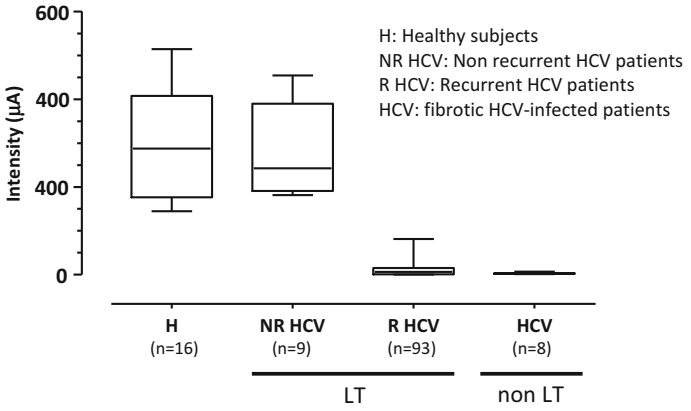

Fig. 3 Comparison of the 5.9 kDa fibrinogen α C-chain fragment levels between HCV patients and healthy subjects. SELDI-TOF-MS intensity values of the 5.9 kDa fibrinogen α C-chain fragment from different groups of HCV patients, nonrecurrent HCV patients, and healthy subjects. The intensity of the peptide released is markedly suppressed in all HCV patients under an active fibrogenic process regardless of liver transplantation (Modified figure and legend from Marfà et al. (2014) and used under the Creative Commons Attribution (CC BY) license. Lack of a 5.9 kDa Peptide C-Terminal Fragment of Fibrinogen a Chain Precedes Fibrosis Progression in Patients with Liver Disease. PLoS ONE 9(10): e109254. doi:10.1371/journal.pone.0109254.g003)

quantified the 5.9 kDa fragment with the use of a homemade ELISA (Noda et al. 2011), confirming previous reports and indicating a clear downregulation of this fragment in serum samples from HCV patients with different stages of fibrosis. Later, in a study focused on the identification of early circulating serum biomarkers of active fibrogenesis, Marfà et al. (2014) also identified the same 5.9 kDa fragment of the fibrinogen α C-chain by the SELDI-TOF-MS technique. In fact, this investigation took advantage of the faster recurrence of hepatic fibrosis that usually occurs in HCV patients submitted to liver transplantation (LT). A marked downregulation of this fragment was detected in serum samples from ten HCV-positive patients submitted 6 months after LT surgery compared to nine LT patients without HCV-RNA recurrence who also underwent antiviral treatment before surgery. It is of note that at 6 months after LT most of these patients showed no evidence of hepatic fibrosis. In addition, all patients underwent a liver biopsy 1 year after LT. Forty percent of the cohort had a fibrosis stage lower than two according to Scheuer classification (Scheuer 1995), indicating that the 5.9 kDa fragment of fibrinogen α C-chain seems to be altered long before any histological evidence is available, thereby enhancing its relevance as a biomarker of early fibrogenic processes. In the same study, 83 HCV LT patients were also analyzed in order to validate the results obtained, and the outcomes confirmed that this fragment behaved as an early serum biomarker of fibrosis. Furthermore, the 5.9 kDa peptide was also determined in serum samples from eight HCV non-LT patients with different degrees of fibrosis, and the results were identical (Fig. 3).

5.9 kDA Fibrinogen Alpha C-Chain Fragment Behavior in Other Hepatic Etiologies

The serum levels of the 5.9 kDa fragment of the fibrinogen α C-chain were also evaluated in samples from patients with etiologies other than HCV infection and ALD, such as NASH, HBV, autoimmune hepatitis (AH), and cryptogenic liver damage (Marfà et al. 2014) (Fig. 4). The results of this investigation were in line with those obtained in HCV-infected patients and in ALD patients, since the absence of the 5.9 kDa fragment was confirmed in all these subjects. Therefore, the lack of the 5.9 kDa peptide is a common finding in patients with an active fibrogenic process regardless of whether this is the consequence of altered lipid metabolism, alcohol intake, or viral infection (Fig. 5). In addition to this and in contrast to other previously described biomarkers of liver fibrosis, the 5.9 kDa fragment of the fibrinogen α C-chain could be useful to identify those subjects under an active fibrogenic process including those in an early stage of liver damage rather than to stage fibrotic patients.

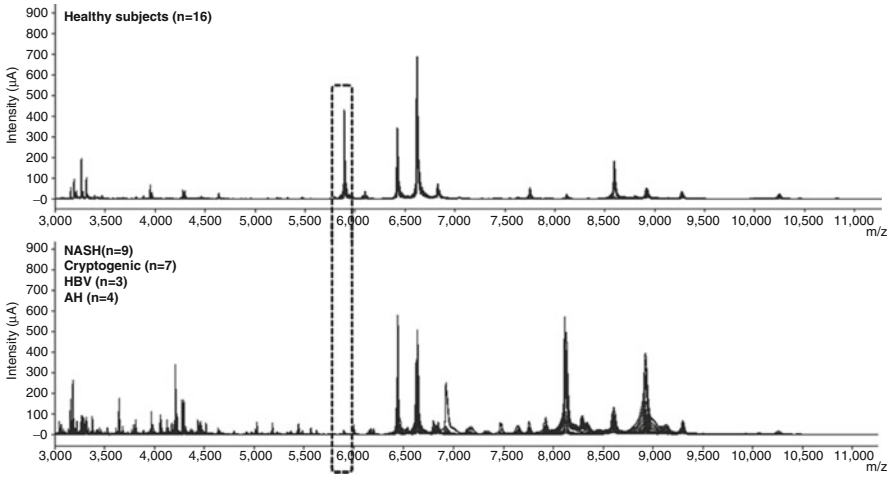

Fig. 4 Lack of the 5.9 kDa fibrinogen α C-chain fragment in fibrotic patients. Fragment of the SELDI-TOF-MS spectra ranging from 3,000 to 11,000 m/z of serum samples obtained from 16 healthy subjects and 23 patients with liver fibrosis of several etiologies including NASH, AH, HBV, and cryptogenic. The lack of the 5.9 kDa fibrinogen α C-chain fragment is clearly associated with fibrogenesis regardless of its etiology (Modified figure and legend from Marfà et al. (2014) and used under the Creative Commons Attribution (CC BY) license. Lack of a 5.9 kDa Peptide C-Terminal Fragment of Fibrinogen a Chain Precedes Fibrosis Progression in Patients with Liver Disease. PLoS ONE 9(10): e109254. doi:10.1371/journal.pone.0109254.g002)

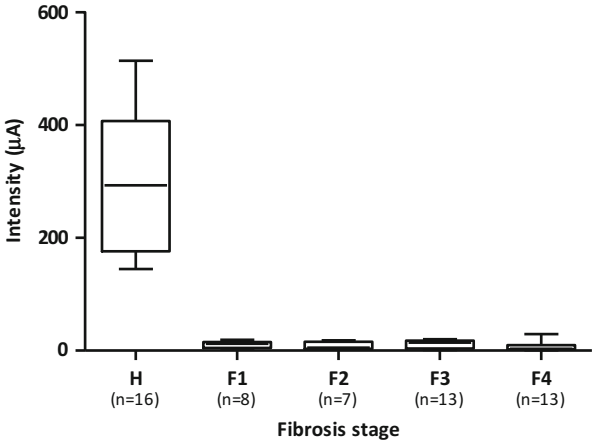

Fig. 5 Comparison of the 5.9 kDa fibrinogen α C-chain fragment levels among stages of liver fibrosis. SELDI-TOF-MS intensity values of the 5.9 kDa fibrinogen α C-chain fragment between patients with different stages of liver fibrosis and healthy subjects (H). The intensity of the released peptide was clearly suppressed in all fibrotic patients including subjects with an early fibrogenic process (F1) (Figure obtained from the data from Marfà et al. (2014) and used under the Creative Commons Attribution (CC BY) license. Lack of a 5.9 kDa Peptide C-Terminal Fragment of Fibrinogen a Chain Precedes Fibrosis Progression in Patients with Liver Disease. PLoS ONE 9(10): e109254. doi:10.1371/journal.pone.0109254)

Cell Regulation and Serum Stability of the 5.9 kDA Fibrinogen Alpha C-Chain Fragment

The mechanisms by which the 5.9 kDa fragment of the fibrinogen α C-chain is diminished in serum samples from patients with an active fibrogenic process are not fully understood. It is well established that IL-6 is a major regulator of fibrinogen gene expression (Fuller and Zhang 2001), suggesting that mediators involved in inflammatory response could be responsible for the altered serum content of the 5.9 kDa fragment in patients with liver fibrosis. To test this hypothesis, Marfà et al. (2014) recently exposed HepG2 cells, a human liver hepatocellular carcinoma cell line, to several well-known profibrogenic and proinflammatory agents including TNF-α, lipopolysaccharide, angiotensin II, endothelin-1, apelin, fibronectin, interleukin-1β, and TGF-β. Of note was that only TGF-β was able to reduce the amount of the 5.9 kDa fragment in the cell culture medium of these cells. This was associated with a downregulated mRNA expression of the fibrinogen α chain gene (FGA) but not of the transcripts corresponding to the β and γ chain genes (FGB and FGG, respectively) (Fig. 6). This was the first experimental data demonstrating that TGF-β is a specific regulator of the 5.9 kDa fragment.

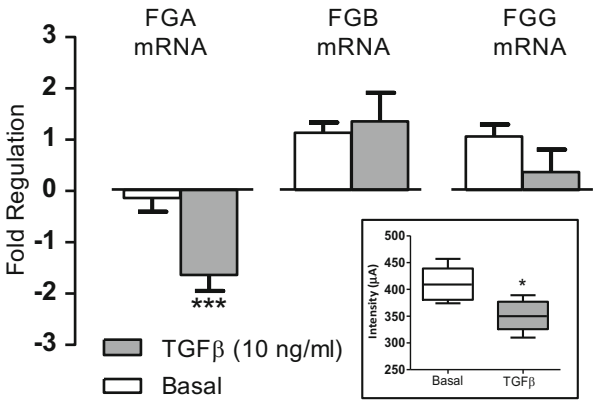

Fig. 6 TGF-β reduces fibrinogen α chain expression in HepG2 cells. Fold regulation in fibrinogen alpha chain (FGA), beta chain (FGB), and gamma chain (FGG) gene regulation in HepG2 cells after 6 h of treatment with TGF-β (10 ng/ml). Results are given as mean ± SE; ***$p < 0.001$ versus basal. Statistical analysis was calculated by the unpaired student's t test. The insert shows the intensity values of the 5.9 kDa peak detected in the cellular supernatant of HepG2 cells after 48 h of treatment with TGF-β (10 ng/ml). Results are given as mean ± SE; *$p < 0.05$ versus basal. Statistical analysis was calculated by the Mann-Whitney U test (Original figure and legend from Marfà et al. (2014) and used under the Creative Commons Attribution (CC BY) license. Lack of a 5.9 kDa Peptide C-Terminal Fragment of Fibrinogen a Chain Precedes Fibrosis Progression in Patients with Liver Disease. PLoS ONE 9(10): e109254. doi:10.1371/journal.pone.0109254.g005)

Demographic variables as well as preanalytical conditions are major issues for considering the diagnostic feasibility of any biomarker. In this regard Sogawa et al. (2013) demonstrated that gender and age did not affect the serum levels of the 5.9 KDa fragment (Fig. 7) but it is modified by factors such as the interval between venipuncture and serum separation. These results indicate that sample processing conditions should be accurately established in order to avoid analytical misinterpretations.

Potential Applications to Prognosis, Other Diseases, or Conditions

At present there is no study specifically addressed at estimating the strength of the 5.9 kDa fibrinogen α C-chain fragment as a prognostic or diagnostic tool in liver disease. On the other hand, taking this biomarker from the research lab to the daily routine in the clinical diagnostic lab depends not only on accurately defining the sensitivity, specificity, positive predictive value, and negative predictive value as indicators of early fibrogenic activity but also determining the availability of high-throughput methods, such as automatic immunoassays, to measure its concentration in biological fluids.

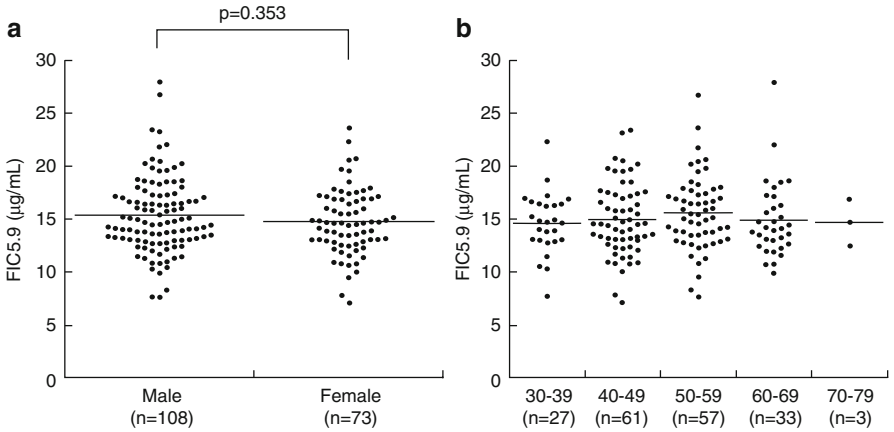

Fig. 7 Serum levels of the 5.9 kDa fibrinogen α C-chain fragment according to age and gender. 5.9 kDa fibrinogen α C-chain fragment concentration based on gender (**a**) and age (**b**). No differences were observed between groups (Figure from Sogawa et al. (2013) with permission from the Publisher John Wiley and Sons, Inc)

Finally, neither is it known whether the 5.9 kDa fragment is a specific biomarker for liver fibrosis. In this regard, a study performed by Pang et al. (2006) showed that the MS spectra of patients with severe acute respiratory syndrome (SARS) during the outbreak period displayed a diminution in the intensity of the 5.9 kDa fragment in comparison to non-SARS patients.

The SELDI-TOF-MS proteomic technique has also been widely used to investigate altered expression of the 5.9 kDa fragment in other pathologies including different types of neoplasia. Several experimental data have suggested that this fragment could be a biomarker in this condition. For instance, a diminution in the same m/z intensity of the fragment has been described in breast, ovarian, prostate, and non-small cell lung cancers (Engwegen et al. 2006; Belluco et al. 2007). In contrast, increased intensities were found in colorectal cancer and pancreatic adenocarcinoma (Koopmann et al. 2004; Engwegen et al. 2006). It should be noted that none of these investigations identified the amino acid sequence of the protein peak. However, it is presumably the same 5.9 kDa fragment of fibrinogen α C-chain since the sample processing and MS methods were very similar to those investigations in which the amino acid sequence identification was performed. Therefore, it is quite plausible that the 5.9 kDa fragment of fibrinogen α C-chain can also be useful as a biomarker in some types of neoplastic diseases.

Summary Points

- Until the last decade, invasive techniques were the only option to stage liver fibrosis.

- Recent technological advances have allowed the development of new imaging techniques and the identification of novel circulating protein markers to detect and monitor the evolution of liver diseases.
- Although many circulating biomarkers are appropriate for the identification and follow-up of the severe stages of liver fibrosis, their diagnostic capacity is lower in early and mild stages.
- The 5.9 kDa fragment of the fibrinogen α C-chain detected in serum is able to identify patients in early stages of liver fibrosis.
- No differences were observed between fibrotic patients in terms of age, gender, etiology, or fibrosis stage when the 5.9 kDa fragment of the fibrinogen α C-chain was evaluated.
- TGF-β seems to be one of the potential stimuli that affect the release of this 5.9 kDa fibrinogen peptide.

Acknowledgments Part of the work described in this chapter has been supported by grants from Dirección General de Investigación Científica y Técnica (SAF 2009-08839 and 2012-35979 to W. Jiménez and BES-2010-035452 to S. Marfà) and co-financed by the European Union through the European Regional Development Fund (ERDF) "A way of making Europe." CIBEREHD is funded by the Instituto de Salud Carlos III.

References

Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003;422:198–207.

Alrawashdeh W, Crnogorac-Jurcevic T. Biomarker discovery in biological fluids. In: Ivanov AR, Lazarev AV, editors. Sample preparation in biological mass spectrometry. 1st ed. New York: Springer; 2011. p. 291–326.

Anton RF, Lieber C, Tabakoff B, et al. Carbohydrate-deficient transferrin and gamma-glutamyltransferase for the detection and monitoring of alcohol use: results from a multisite study. Alcohol Clin Exp Res. 2002;26:1215–22.

Baranova A, Lal P, Birerdinc A, et al. Non-invasive markers for hepatic fibrosis. BMC Gastroenterol. 2011;11:91.

Bataller R, Brenner DA. Liver fibrosis. J Clin Invest. 2005;115:209–18.

Bedossa P, Dargère D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. Hepatology. 2003;38:1449–57.

Belluco C, Petricoin EF, Mammano E, et al. Serum proteomic analysis identifies a highly sensitive and specific discriminatory pattern in stage 1 breast cancer. Ann Surg Oncol. 2007;14:2470–6.

Camerini S, Mauri P. The role of protein and peptide separation before mass spectrometry analysis in clinical proteomics. J Chromatogr A. 2015;1381:1–12.

Carrión JA, Fernández-Varo G, Bruguera M, et al. Serum fibrosis markers identify patients with mild and progressive hepatitis C recurrence after liver transplantation. Gastroenterology. 2010;138:147–158.e1.

Castera L. Noninvasive methods to assess liver disease in patients with hepatitis B or C. Gastroenterology. 2012;142:1293–1302.e4.

Castera L, Pinzani M. Biopsy and non-invasive methods for the diagnosis of liver fibrosis: does it take two to tango? Gut. 2010;59:861–6.

Castera L, Forns X, Alberti A. Non-invasive evaluation of liver fibrosis using transient elastography. J Hepatol. 2008;48:835–47.

Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol. 2005;129:307–21.

Crespo G, Fernández-Varo G, Mariño Z, et al. ARFI, FibroScan, ELF, and their combinations in the assessment of liver fibrosis: a prospective study. J Hepatol. 2012;57:281–7.
Davalos D, Akassoglou K. Fibrinogen as a key regulator of inflammation in disease. Semin Immunopathol. 2012;34:43–62.
de Maat MP, Nieuwenhuizen W, Knot EA, et al. Measuring plasma fibrinogen levels in patients with liver cirrhosis. The occurrence of proteolytic fibrin(ogen) degradation products and their influence on several fibrinogen assays. Thromb Res. 1995;78:353–62.
Dimmitt SB, Rakic V, Puddey IB, et al. The effects of alcohol on coagulation and fibrinolytic factors: a controlled trial. Blood Coagul Fibrinolysis. 1998;9:39–45.
Doolittle RF. Structural aspects of the fibrinogen to fibrin conversion. Adv Protein Chem. 1973;27:1–109.
Engwegen JY, Helgason HH, Cats A, et al. Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry. World J Gastroenterol. 2006;12:1536–44.
Fernandez-Varo G, Jimenez W. Non invasive markers of liver fibrosis. Eur Gastroenterol Hepatol Rev. 2011;7:93–6.
Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet. 2012;379:1245–55.
Friedman SL. Evolving challenges in hepatic fibrosis. Nat Rev Gastroenterol Hepatol. 2010;7:425–36.
Fuller GM, Zhang Z. Transcriptional control mechanism of fibrinogen gene expression. Ann N Y Acad Sci. 2001;936:469–79.
Gorinstein S, Caspi A, Goshev I, et al. Structural changes in plasma circulating fibrinogen after moderate beer consumption as determined by electrophoresis and spectroscopy. J Agric Food Chem. 2003;51:822–7.
Heidelbaugh JJ, Bruderly M. Cirrhosis and chronic liver failure: part I. Diagnosis and evaluation. Am Fam Physician. 2006;74:756–62.
Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. Annu Rev Pathol. 2011;6:425–56.
Herrick S, Blanc-Brude O, Gray A, et al. Fibrinogen. Int J Biochem Cell Biol. 1999;31:41–6.
Hortin GL. The MALDI-TOF mass spectrometric view of the plasma proteome and peptidome. Clin Chem. 2006;52:1223–37.
Koopmann J, Zhang Z, White N, et al. Serum diagnosis of pancreatic adenocarcinoma using surface-enhanced laser desorption and ionization mass spectrometry. Clin Cancer Res. 2004;10:860–8.
Lee UE, Friedman SL. Mechanisms of hepatic fibrogenesis. Best Pract Res Clin Gastroenterol. 2011;25:195–206.
Lichtinghagen R, Pietsch D, Bantel H, et al. The Enhanced Liver Fibrosis (ELF) score: normal values, influence factors and proposed cut-off values. J Hepatol. 2013;59:236–42.
Marfà S, Crespo G, Reichenbach V, et al. Lack of a 5.9 kDa peptide C-terminal fragment of fibrinogen α chain precedes fibrosis progression in patients with liver disease. PLoS One. 2014;9:e109254.
Martinez SM, Fernández-Varo G, González P, et al. Assessment of liver fibrosis before and after antiviral therapy by different serum marker panels in patients with chronic hepatitis C. Aliment Pharmacol Ther. 2011;33:138–48.
Matsuda Y, Tsuchishima M, Ueshima Y, et al. The relationship between the development of alcoholic liver and pancreatic diseases and the induction of gamma glutamyl transferase. Alcohol Alcohol Suppl. 1993;1B:27–33.
Mistry PK, Jain D. Haematological disorders of the liver. In: Dooley J, Lok A, Burroughs A, Heathcote J, editors. Sherlock's diseases of the liver and biliary system. 12th ed. Oxford: Wiley-Blackwell; 2011. p. 48–69.
Mosesson MW, Siebenlist KR, Meh DA. The structure and biological features of fibrinogen and fibrin. Ann N Y Acad Sci. 2001;936:11–30.
Noda K, Sogawa K, Kikuchi W, et al. Development of a sandwich ELISA for the 5.9-kDa fibrinogen alpha C chain fragment detected by serum proteome analysis. Proteomics Clin Appl. 2011;5:141–6.

Nomura F, Tomonaga T, Sogawa K, et al. Identification of novel and downregulated biomarkers for alcoholism by surface enhanced laser desorption/ionization-mass spectrometry. Proteomics. 2004;4:1187–94.
Pang RT, Poon TC, Chan KC, et al. Serum proteomic fingerprints of adult patients with severe acute respiratory syndrome. Clin Chem. 2006;52:421–9.
Pluta A, Gutkowski K, Hartleb M. Coagulopathy in liver diseases. Adv Med Sci. 2010;55:16–21.
Rosenberg WM, Voelker M, Thiel R, European Liver Fibrosis Group, et al. Serum markers detect the presence of liver fibrosis: a cohort study. Gastroenterology. 2004;127:1704–13.
Scheuer PJ. The nomenclature of chronic hepatitis: time for a change. J Hepatol. 1995;22:112–4.
Schuppan D, Kim YO. Evolving therapies for liver fibrosis. J Clin Invest. 2013;123:1887–901.
Sebastiani G, Vario A, Guido M, et al. Stepwise combination algorithms of non-invasive markers to diagnose significant fibrosis in chronic hepatitis C. J Hepatol. 2006;44:686–93.
Sierksma A, van der Gaag MS, Kluft C, et al. Moderate alcohol consumption reduces plasma C-reactive protein and fibrinogen levels; a randomized, diet-controlled intervention study. Eur J Clin Nutr. 2002;56:1130–6.
Sogawa K, Itoga S, Tomonaga T, et al. Diagnostic values of surface-enhanced laser desorption/ionization technology for screening of habitual drinkers. Alcohol Clin Exp Res. 2007;31:S22–6.
Sogawa K, Satoh M, Kodera Y, et al. A search for novel markers of alcohol abuse using magnetic beads and MALDI-TOF/TOF mass spectrometry. Proteomics Clin Appl. 2009;3:821–8.
Sogawa K, Kodera Y, Noda K, et al. The measurement of a fibrinogen α C-chain 5.9 kDa fragment (FIC 5.9) using MALDI-TOF MS and a stable isotope-labeled peptide standard dilution. Clin Chim Acta. 2011;412:1094–9.
Sogawa K, Noda K, Umemura H, et al. Serum fibrinogen alpha C-chain 5.9 kDa fragment as a biomarker for early detection of hepatic fibrosis related to hepatitis C virus. Proteomics Clin Appl. 2013;7:424–31.
Takahashi H, Tatewaki W, Wada K, et al. Fibrinolysis and fibrinogenolysis in liver disease. Am J Hematol. 1990;34:241–5.
Tennent GA, Brennan SO, Stangou AJ, et al. Human plasma fibrinogen is synthesized in the liver. Blood. 2007;109:1971–4.
Tsochatzis EA, Bosch J, Burroughs AK. Liver cirrhosis. Lancet. 2014;383:1749–61.
van Golde PM, Hart HC, Kraaijenhagen RJ, et al. Regular alcohol intake and fibrinolysis. Neth J Med. 2002;60:285–8.
Watkins PB. Biomarkers for the diagnosis and management of drug-induced liver injury. Semin Liver Dis. 2009;29:393–9.

YKL-40 as a Biomarker of Liver Diseases

24

Salvatore Musumeci

Contents

Abstract

YKL-40 association with human disease has been the object of many years of investigation. Increased YKL-40 protein expression is noted in patients with alcoholic liver disease and concurrent chronic hepatitis C virus infection. Patients with alcoholic cirrhosis, posthepatitic cirrhosis, and non-cirrhotic fibrosis had significantly higher serum YKL-40 than normal subjects. Serum YKL-40 was significantly related to the degree of liver fibrosis with the highest levels in patients with moderate to severe fibrosis. Large prospective studies of patients with liver diseases are needed to determine if patients with slight liver fibrosis and high serum YKL-40 are at risk of developing cirrhosis. It has, also, been suggested that YKL-40 has a role in cancer cell growth and survival, participating in the inflammatory process around the tumor and in angiogenesis. The highest serum YKL-40 levels were found in patients with metastatic tumor with poorest prognosis. Potential molecules have been proposed to inhibit YKL-40 activity

S. Musumeci (✉)
Department of Chemical Sciences, University of Catania and Institute of Biomolecular Chemistry, CNR, Catania, Italy
e-mail: smusumeci@tiscalinet.it

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_21

such as siRNA, human (or humanized) monoclonal antibodies specific for YKL-40 or its receptor(s), YKL-40 receptor antagonists, or substrate molecules that competitively bind to YKL-40. Such YKL-40 could be a helpful serum marker to estimate the degree of liver fibrosis, and its inhibition could be expected to have therapeutic efficacy in liver disease.

Keywords
YKL-40 • Liver steatosis • Liver fibrosis • Liver metastasis

List of Abbreviations

%	Percentage
AMCase	Acidic mammalian chitinase
AFP	Alpha-fetoprotein
CHI3L1	Chitinase-3-like-1
ELISA	Enzyme-linked immunoassay
EGF	Epidermal growth factor
ESR	Erythrocyte sedimentation rate
HA	Hyaluronic acid
HSC	Hepatic stellate cell
HCV	Hepatitis C virus
h	Hour
IGF-1	Insulin-like growth factor-1
IFNγ	Interferon-γ
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
MRI	Magnetic resonance imaging
MHC	Major histocompatibility complex
NAFLD	Non-alcoholic fatty liver disease
RT-PCR	Reverse transcriptase polymerase chain reaction
TMUGS	Tumor marker utility grading system
TNF α	Tumor necrosis factor alpha

Introduction

Through the years, the association of the chitinase YKL-40 with human pathology has been greatly studied. The 18 glycosyl hydrolase is a chitinase belonging to a family of genes in prokaryotic cells that is also expressed in eukaryotic cells. In mammals, despite the absence of endogenous chitin, a number of chitinases and chitinase-like proteins have been identified although their role is yet to be fully identified. YKL-40 (also known as chitinase 3-like 1) is a glycoprotein that belongs to this family. The YKL-40 gene is located on chromosome 1q32.1 and two different splice forms are reported: isoform 1 containing exon 1–10 and isoform 2 in which exon 8 has been spliced out (Rehli et al. 1997). It is involved in apoptosis, enhances adaptive Th2

immunity, stimulates macrophage activation, inhibits oxidant-induced lung injury, and participates to fibrotic processes and wound healing (Johansen 2006).

YKL-40 was found expressed by macrophage during the late stages of differentiation (Rehli et al. 2003), by infiltrating macrophages in several inflammatory conditions such as osteoarthritis and rheumatoid arthritis (Volck et al. 2001), by tumor-associated macrophages (Junker et al. 2005), and by macrophage and giant cells in vessels affected by arteritis (Johansen et al. 1999). YKL-40 is also enhanced in macrophages of early atherosclerotic lesions (Boot et al. 1999) and of bronchial tissue (Chupp et al. 2007).

YKL-40 protein expression is also increased in patients with alcoholic liver disease and concurrent chronic hepatitis C virus infection (Johansen et al. 1997; Kamal et al. 2006). YKL-40 is not expressed in normal liver tissue except in the mesenchymal structure of the portal tract (Pinzani 1999). Interestingly, in chronic viral hepatitis, portal tracts are the primary sites of fibrotic tissue formation from which fibrotic liver disease will initiate the changes in liver architecture associated with the infection.

YKL-40 expression is associated with several pathologies, but its biological role is still poorly understood.

YKL-40 Expression in the Liver

Hakala et al. (1993) reported that YKL-40 mRNA was strongly expressed in human liver tissue. Subsequently, Hu et al. (1996) was unable to demonstrate YKL-40 mRNA expression in normal liver tissue. Immunohistochemical analysis of liver biopsies, from patients with various liver diseases, has shown YKL-40 protein expression in areas with slight fibrosis (either pericellular or perisinusoidal), along fibrotic septa, in association with signs of fibrogenesis and in areas with moderate and severe fibrosis (Johansen et al. 1997) (Figs. 1, 2, and 3).

It is possible that in the study by Hakala et al. (1993) the tissue analyzed could be derived from fibrotic tissue, resulting in high expression of YKL-40.

Hepatocytes did not express YKL-40 protein and no expression was found in normal liver tissue except in the mesenchymal structures of the portal tract. Patients with chronic active HCV had YKL-40 protein expression in areas with the presence of necrosis, but not in the lymphocytes. YKL-40 protein expression was found in 90% of liver biopsies with moderate or severe fibrosis, in 86% of those with slight fibrosis, and in 93% of biopsies that presented fibrogenesis. YKL-40 expression was found in extracellular matrix (ECM) in both areas free of cells as well as in areas with cells (Johansen et al. 1997). Since YKL-40 is a growth factor for fibroblasts, working synergistically with IGF-1 (Recklies et al. 2002), it may play a role in the pathological conditions leading to liver fibrosis. Shackel et al. (2003) showed, using suppression subtractive hybridization and quantitative real-time RT-PCR, that YKL-40 was one of the most differentially expressed genes in liver tissue from end-stage cirrhosis due to HCV, compared to non-diseased liver tissue, primary biliary cirrhosis, and autoimmune hepatitis-associated cirrhosis (Shackel et al. 2003).

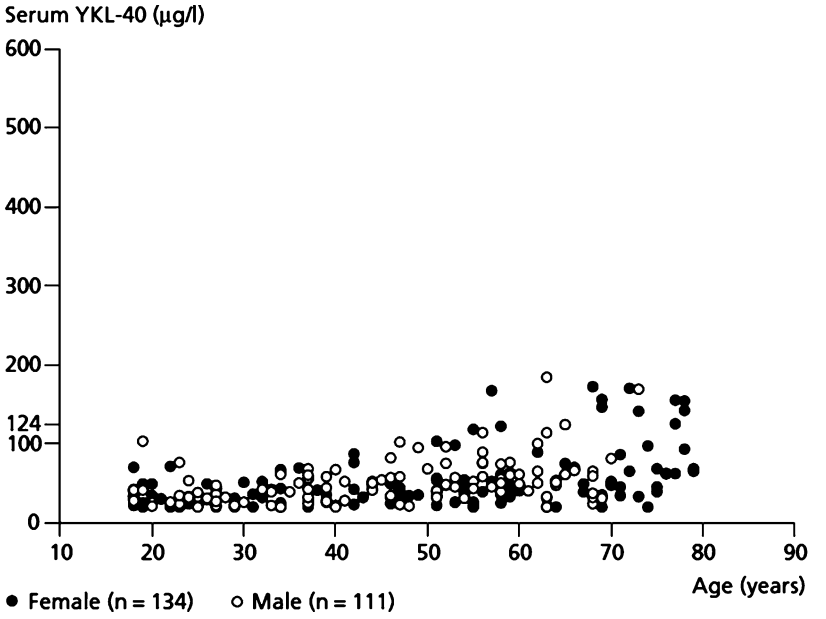

Fig. 1 Serum YKL-40 concentration in healthy adult in relation to sex and age. The upper 95th percent limit of serum YKL-40 levels in these healthy adults is 124 µg/l. From Julia Johansen studies on serum YKL-40 as a biomarker in disease with inflammation, tissue remodeling, fibroses, and cancer. Danish Medical Bulletin Vol 53 Pages 172–209

YKL-40 and Liver Fibrosis

Liver fibrosis is a dynamic and complex process where excessive accumulation of extracellular matrix proteins occurs, characterizing chronic liver disease. Liver fibrosis progresses rapidly to cirrhosis in several clinical settings, leading to liver failure and hypertension up to liver transplantation. Percutaneous liver biopsy is considered the gold-standard method for the assessment of liver fibrosis and fibrogenesis. Liver biopsy is an invasive procedure, but necessary because, up to day, biochemical and serological tests are of little importance for evaluating intermediate grades of fibrosis and the activity of fibrogenesis. YKL-40 belongs to a group of proteins like human chitinase, free of chitinolytic activities. YKL-40 mRNA appears to be elevated in diseases such as liver fibrosis and malignant tumors and is secreted by many cells such as macrophages, chondrocytes, and cancer cells. The expression of YKL-40 was found increased in several inflammatory diseases. So YKL-40 may be considered an inflammatory protein that participates in the endothelial dysfunction by promoting chemotaxis, cell adhesion, and migration, resulting in tissue remodeling in response to endothelial damage. Several studies show the association of high serum levels of YKL-40 and the presence of endothelial damage and even turn out to be higher levels of YKL-40 in liver injury and fibrosis (Tao et al. 2014).

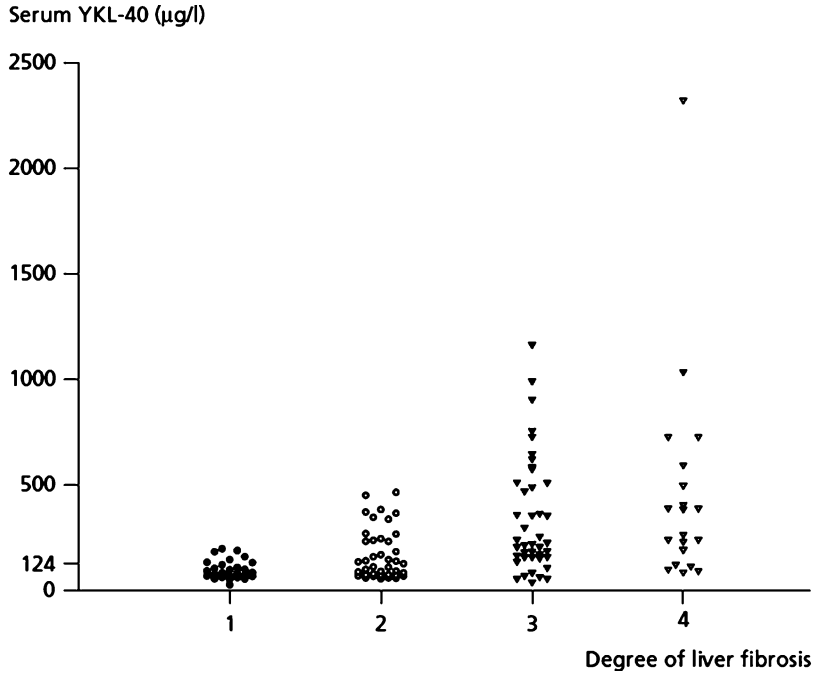

Fig. 2 Individual serum YKL-40 concentration in patients with different degrees of liver fibrosis: *1* = no fibrosis, *2* = slight fibrosis, *3* = moderate fibrosis, *4* = severe fibrosis. The upper 95th percent limit of serum YKL-40 levels in these healthy adults is 124 µg/l. From Julia Johansen studies on serum YKL-40 as a biomarker in disease with inflammation, tissue remodeling, fibroses, and cancer. Danish Medical Bulletin Vol 53 Pages 172–209

Serum levels in various situations are displayed in Figs. 1–3. YKL-40 level in serum related to histological findings and immunohistochemical staining for YKL-40 in liver was analyzed in patients with suspected liver disease, at the same time. The median serum concentrations of YKL-40 were higher in patients with alcoholic cirrhosis (532 µ/l) and especially in patients with additional alcoholic hepatitis (740 µ/l). Patients with alcoholic cirrhosis, post-hepatitis cirrhosis (425 µ/l), and non-cirrhotic fibrosis (330 µ/l) had significantly higher serum YKL-40 levels than normal subjects (102 µ/l) and patients with nonalcoholic fatty liver (195 µ/l).

Serum YKL-40 had a significant ($p < 0.001$) correlation to the degree of liver fibrosis, with the highest levels present in patients with moderate (466 µ/l) to severe (676 µ/l) fibrosis. An increase of serum YKL-40 ($p = 0.018$) was also found in patients with slight fibrosis (270 µ/l), while no increase was noted in patients without fibrosis. Immunohistochemical analysis demonstrated positive staining for the YKL-40 antigen in areas with fibrosis, particularly in areas with active fibrogenesis but not in hepatocytes. This study therefore indicates that the increased serum levels of YKL-40, in patients with liver disease of various degrees, seem to reflect fibrosis and fibrogenesis (Johansen et al. 2000).

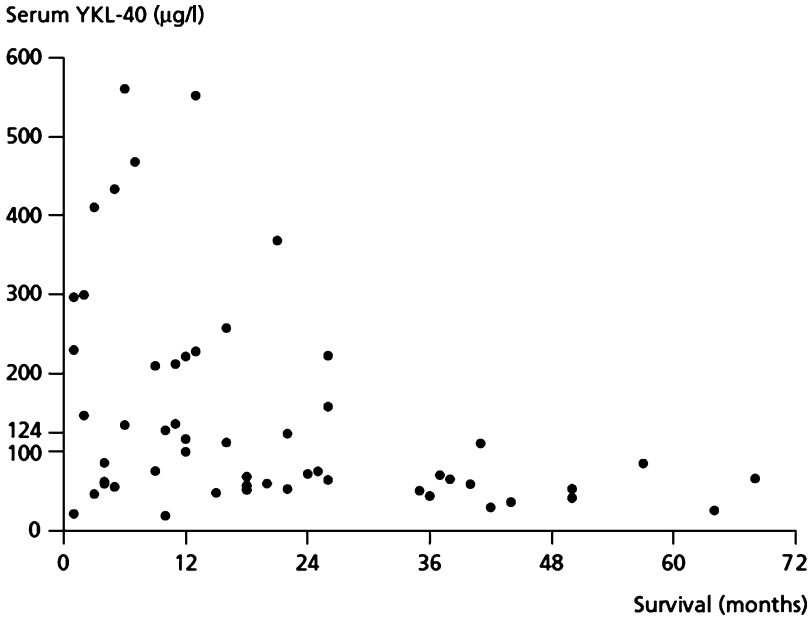

Fig. 3 Individual serum YKL-40 concentration in patients with metastatic breast cancer in relation to months of survival. The upper 95th percent limit of serum YKL-40 levels in these healthy adults is 124 µg/l. From Julia Johansen studies on serum YKL-40 as a biomarker in disease with inflammation, tissue remodeling, fibroses, and cancer. Danish Medical Bulletin Vol 53 Pages 172–209

YKL-40 and HCV Infection

YKL-40 has been evaluated as a clinical marker in patients with HCV-associated liver disease. One hundred and nine patients with HCV-associated liver disease were enrolled, and serum type IV collagen, amino-terminal peptide of type III procollagen (PIIIP), HA, and YKL-40 levels were analyzed through RIA or ELISA. Sixty-seven of 109 patients received interferon (IFN) therapy, while 88 underwent liver biopsy. The authors, in particular, investigated the relationship between the level of serum fibrosis markers and histological fibrosis scores, analyzing the levels of fibrosis markers before and after IFN therapy. The increase in serum concentration of all markers, particularly HA, was correlated with the progression of liver fibrosis. YKL-40 had a strong correlation with HA ($r = 0.536$, $P < 0.0001$). This study highlighted that YKL-40 was superior to other fibrosis markers for predicting severe fibrosis from mild fibrosis (YKL-40, AUC = 0.809; HA, AUC = 0.805). In particular, after IFN therapy, only YKL-40 values significantly decreased not only in the responder group but also in the nonresponder group ($P = 0.03$) (Saitou et al. 2005).

Studies have been conducted on promoter polymorphism CHI3L1, encoding YKL-40, to assess its eventual association with HCV-induced liver fibrosis.

Moreover, the influence of this polymorphism on the serum concentrations of YKL–40 was also evaluated. CHI3L1 promoter polymorphism was found in 440 patients affected by HCV infection, with 5′-endonuclease assays. The authors also found the association between a lower stage of liver fibrosis with lower serum YKL-40 levels in HCV patients (Berres et al. 2009).

When Fontana et al. (2012) explored the association of this YKL-40 promoter polymorphism in the hepatitis C antiviral long-term treatment against cirrhosis (HALT-C), they found, among these patients, an association between the baseline liver disease severity with the risk of liver disease progression suggesting that this polymorphism is not associated with disease progression in chronic hepatitis patients with established fibrosis. In light of these statements, probably YKL-40 promoter polymorphism is associated with the severity of HCV-related liver disease in patients, who have other risk factors or cofactors for environmental advanced fibrosis. Among other things, this locus could be more important when considering the establishment or development of fibrosis rather than early in the progression of more advanced or in stable liver fibrosis.

YKL-40 and Non-alcoholic Fatty Liver Disease

NAFLD patients reflect pathological conditions involving micro- and macro-vesicular steatosis in hepatocytes. In particular, this condition has the highest risk for progressing to end-stage liver disease.

In a significant study, confirmed NAFLD patients were divided in two groups in order to construct (n = 480) and validate (n = 253) a scoring system. Different variables were analyzed by multivariable modeling in order to predict the presence or absence of advanced fibrosis. Age, body mass index, and laboratory parameters such as AST/ALT ratio, hyperglycemia, platelet count, and albumin were independent factors of advanced liver fibrosis. The scoring system that considered these variables had a characteristic area under the receiver operating curve of 0.88 and 0.82 in. respectively, in the estimation and validation groups. The application of a high cutoff score (0.676) ensured so the presence of advanced fibrosis could be diagnosed with great accuracy (positive predictive value of 90% and 82% in the estimation and validation groups, respectively). Liver biopsy would have been avoided in 549 (75%) of the 733 patients, by applying this model, with a correct prediction in 496 patients (90%) (Table 1).

This easy model accurately separates patients affected by NAFLD in subjects with and without advanced fibrosis. Thus, liver biopsy for identifying advanced fibrosis is unnecessary in a great percentage of patients (Angulo et al. 2007).

Lebensztejn et al. (2011) suggested to replace liver biopsy with serum markers that can predict the degree of liver fibrosis in fatty liver disease. Serum levels of HA, laminin, YKL-40, and cytokeratin-18 M30 (CK18M30) were analyzed in a sample of 52 children with NAFLD in which 19 of them were diagnosed with liver fibrosis.

Table 1 Serum YKL-40 in patients with liver diseases (values are median range)

(μg/l)	n	Serum YKL-40		High YKL-40 (%)	
Fatty liver[a]	16	93	(24–195)	25	Johansen et al. 2000a VI
Viral hepatitis[a]	17	83	(53–182)	35	
Non-cirrhotic fibrosis[a]	31	158[b]	(55–463)	61	
Posthepatitic cirrhosis[a]	10	204[b]	(69–992)	80	
Alcoholic cirrhosis[a]	51	255[b]	(39–2323)	90	
Chronic hepatitis C	49	78[b]	(18–1276)	53	Nøjgaard et al. 2003b
Alcoholics, no fibrosis	17	147	(550)°	–	Tran et al. 2000
Alcoholics, mild fibrosis	55	158	(800)°	–	
Alcoholics, moderate fibrosis	15	402	(1,500)°	–	
Alcoholics, severe fibrosis	59	511	(1,600) °	–	
Alcoholics, no fibrosis[a]	43	72	(10–388)	26	Nøjgaard et al. 2003a
Alcoholics, slight fibrosis[a]	88	156[b]	(31–2,658)	64	
Alcoholics, moderate fibrosis[a]	146	186[b]	(38–2,658)	75	
Alcoholics, severe fibrosis[a]	59	201[b]	(38–1,532)	76	

From Julia Johansen studies on serum YKL-40 as a biomarker in disease with inflammation, tissue remodeling, fibroses, and cancer. Danish Medical Bulletin Vol 53 Pages 172–209

Serum HA and CK18M30 were significant (AUC = 0.672 and 0.666, respectively) for children differentiating fibrosis from those with no fibrosis. The use of both markers is better than laminin and YKL-40, whose levels do not allow to use them as markers of prediction (Lebensztejn et al. 2011).

In a recent study, the level of the biomarker YKL-40 for NAFLD in World Trade Center particulate matter (WTC-PM)-exposed Fire Department of New York (FDNY) rescue workers was analyzed. NAFLD was present in 29/131 (22%) of cohort, but liver biopsy confirmation was not defined by a liver/spleen attenuation ratio ≤ 1.

In a multivariable model, the increase of YKL-40 appears to be protective, while increased triglycerides and alkaline phosphatase are risk factors to NAFLD; however, the results of this study show advantages but also several limitations. The problem is that the serum biomarkers were measured within 3 months after acute WTC-PM, but the CT was performed 5 years after the analysis of the serum and not at the time when the serum was analyzed.

In the light of the above, this study must be completed by comparing the results with an unexposed control group to determine the direct effect of WTC-PM exposure on serum biomarkers (Cho et al. 2014).

Thus, further studies are needed to determine the role of YKL-40 in NAFLD.

YKL-40 and Liver Metastases

Liver metastases denote a poor prognosis often in patients with ovarian, breast, lung, and colorectal carcinoma that may occur months or years after the primary tumor is removed. Elevated serum YKL-40 levels are found in patients with liver metastases (Nøjgaard et al. 2003b; Johansen et al. 1995; Cintin et al. 1999, 2002; Dehn et al. 2003; Jensen et al. 2003). The development of liver metastases comprises several steps, exfoliation of cancer cells from the primary site, entry into the portal system, adhesion to the endothelium and subsequent extravasation into the hepatic microvasculature, and increase and formation of glandular or acinar structure in the liver parenchyma. The success of cancer cells to metastasize to the liver depends not only on their characteristic but also on the hepatic microenvironment. The myofibroblast cell presence occurs with cancers of epithelial origin and could contribute to the evolution in metastatic tumors (Smith 1994). Hepatic stellate cells (HSCs) are the only mesenchymal cells existent in the extravascular space of the liver parenchyma. Under several stimuli, they are activated and differentiated into myofibroblasts. HSCs activated reside around the tumor cells in liver metastases from human carcinoma, but the mechanism of interaction between cancer cells and HSC is not well understood. Cancer cells when metastasize to the liver are able to secrete factors that contribute to activate HSCs and to the progression of hepatic metastasis. Furthermore, growth factors released by HSCs enhance proliferation and migration of cancer cells in vitro (Olaso et al. 1997; Shimizu et al. 2000; Lunevicius et al. 2001). HSCs promote the proliferation of hepatocellular carcinoma cell lines that in turn activate and promote proliferation of the HSC cells (Faouzi et al. 1999; Neaud et al. 1997) demonstrating that dual interactions between these cells YKL-40 stimulate fibroblasts in vitro (Recklies et al. 2002). It could be that YKL-40 secreted by metastatic tumor cells in the liver has an effect on myofibroblast cells and that YKL-40 secreted from HSCs promotes cancer cells.

Hayes et al. (1996), In order to suggest recommendations about promising tumor markers progress from the laboratory into the clinic, Hayes et al., (1996) have introduced the “Tumor Marker Utility Grading System” (TMUGS). Serum YKL-40 is considered on the “utility scale + ” or “utility scale +/−.” YKL-40 is not organ or tumor specific, but retrospective clinical studies with different types of cancers indicate that the evaluation of serum YKL-40 may be helpful in the screening and monitoring of cancer patients. Elevated serum concentration of YKL-40 was found in a subgroup of patients with different types of solid carcinoma (including several types of adenocarcinomas, small cell carcinoma, and glioblastoma). In particular, the highest serum YKL-40 levels were found in patients with metastatic tumor with poorest prognosis. Serum YKL-40 levels provided independent information of survival. Serum YKL-40 represents a potential value as a biomarker in the diagnosis and monitoring of solid cancer but needs more studies. Its role as a biomarker in hematological malignancies has to be determined.

The studies of cancer patients regarding serum YKL-40 as a tumor marker are designed as retrospective, including a small cohort with a lower level of evidence. To

assume diagnostic value, serum YKL-40 levels in patients with primary and advanced carcinoma need to be established in large retrospective studies of high quality confirming the association between serum YKL-40 and poor prognosis. After this, the next step would be the promotion of a prospective study from which the benefit of using serum YKL-40 levels in the clinical decision-making process is assessed.

Then, it would be important to understand if YKL-40 could be a potential target for cancer therapy. The biological function of YKL-40 in cancer development and metastases is unknown, and the elucidation of a possible function of YKL-40 in cancer diseases is an important objective of future studies. It has been demonstrated that YKL-40 displays growth factor activity for cell types involved in tissue remodeling processes, and it has been suggested that YKL-40 has a role in cancer cell growth and survival, in the inflammatory process around the tumor, the angiogenesis, and the remodeling of the ECM surrounding the cancer cells. Based on clinical studies of serum YKL-40 levels in cancer patients, one could hypothesize that YKL-40 will prove to have a role in the ability of cancer cells to proliferate, survive, invade, and metastasize and in cancer cell-matrix interactions and in the production of the altered extracellular matrix surrounding the cancer cells.

Metastatic colorectal cancer (mCRC) often gives liver metastases. A large study found an association between plasma levels of YKL-40 in patients with metastatic colorectal cancer and overall survival in the short term. Five hundred sixty-six patients were included in the study NORDIC VII regimen with first-line oxaliplatino based and with or without cetuximab. In 40% of patients, before treatment, the plasma concentration of YKL-40 was high, supporting the idea of an association between high concentrations of YKL-40 before treatment and poor prognosis of patients with metastatic colorectal cancer (Cintin et al. 1996). Despite gap limitations, it can be said that YKL-40, as an inflammatory biomarker may be useful for monitoring patients with mCRC during treatment, but these findings need to be confirmed about the usefulness of such a biomarker for monitoring of cancer progression (Tarpaag et al. 2014).

Recently, YKL-40 has been shown as an important factor and as metastatic regulator of migration and invasion of tumor cells in cancer non-small cell lung cancer (NSCLC), proposing it as a possible therapeutic target in these patients (Jefri et al. 2015). Future studies should clarify the role of YKL-40, to propose it as an attractive target in the design of anticancer therapies. The potential proposed molecules to inhibit the activity of YKL-40 are siRNA, specific monoclonal antibodies, human or humanized YKL-40, or substrates that bind competitively to YKL-40. All of these molecules, in a different way, may limit cancer growth and progression to metastasis, improving patient survival. Thus, YKL-40 could be expected to have therapeutic efficacy in cancer patients with tumors that produce YKL-40. It is therefore of major importance to investigate if YKL-40 could become a target for the development of new cancer therapies.

Conclusions and Future Perspectives

The methods used for the measurement of serum concentrations of YKL-40 reach good levels in terms of reliability, reproducibility, and stability, even if an automated test for the determining of YKL-40 in serum will hopefully be developed in order to standardize and speed up the analysis. Low level of YKL-40 was found in the serum of healthy subjects that probably originates from activated macrophages and neutrophils and increases with older age due, probably, to the presence of low-grade inflammation or an undiagnosed disease that influences serum YKL-40 levels. It is not clear if YKL-40 is cleared by the kidneys, and then future studies are needed to determine the metabolism of circulating YKL-40.

Correlating YKL-40 serum concentrations with other ECM products secreted by HSCs could be helpful in the diagnosis of liver diseases. Few correlations were found between serum YKL-40 and enzymes secreted by hepatocytes (serum aspartate aminotransferase and alkaline phosphatase), while inverse correlations were found between serum albumin and the coagulation factors 2, 7, and 10 (Johansen et al. 1997; Tran et al. 2000; Nøjgaard et al. 2003a). Serum YKL-40 was correlated with other parameters that reflected the degree of liver fibrosis, such as hepatic venous pressure gradient and post-sinusoidal resistance. It was inversely correlated with the clearance of indocyanine green (Johansen et al. 1997).

Nunes et al. (1998) found that serum YKL-40 decreased in patients with chronic HCV, who responded to interferon treatment, and Nøjgaard et al. (2003b) found that patients with chronic HCV treated for 12 months with alpha-interferon and ribavirin had a decrease in serum YKL-40 at 6 months from the end of treatment. In patients who responded to treatment, serum YKL-40 was not related to changes in HCV titre or liver enzymes during 12 months of treatment. The serum YKL-40 level before therapy could not predict whether a patient would respond to treatment, but the nonresponders had unchanged high serum YKL-40 levels during the 12-month treatment period and at 6 months from the end of therapy.

Nøjgaard et al. (2003a) reported that patients with alcohol-induced liver disease and high serum YKL-40 had shorter survival than alcoholics with normal serum YKL-40 (relative risk = 4.24, 95% confidence interval 2.18–8.26, $p < 0.0001$). Multivariable Cox regression analysis, including serum YKL-40 and variables known to have prognostic information of survival in alcoholics (i.e., years of high alcohol intake; serum creatinine; coagulation factors 2, 7, and 10; alkaline phosphatase; and IgM), showed that serum YKL-40 had no independent prognostic value.

Increased YKL-40 mRNA and protein expressions are found in fibrotic liver tissue of patients with alcoholic liver disease and chronic HCV infection. Serum concentrations of YKL-40 are elevated in most patients with moderate to severe liver fibrosis and cirrhosis, independent of disease etiology, and may provide new information of ongoing fibrogenesis in the liver. Patients with alcoholic liver disease and high serum YKL-40 have a poorer prognosis compared to patients with normal serum YKL-40. Large prospective studies of patients with liver diseases are needed

in order to determine if patients with slight liver fibrosis and high serum YKL-40 are at risk of developing cirrhosis and if serum YKL-40 in combination with other biomarkers of liver fibrosis (e.g., serum hyaluronan and PIIINP) can predict the severity of liver fibrosis and be used in monitoring patients with liver fibrosis or cirrhosis. Serum YKL-40 may also be useful for monitoring of patients with liver diseases during anti-fibrotic or antiviral therapy. The biological function of YKL-40 in liver diseases is not yet known and needs to be determined if YKL-40 has a role in the pathogenesis of liver cirrhosis. As it has been found for fibroblasts, YKL-40 may be a growth factor for HSCs and could stimulate their production of collagen. Reducing the ECM production by activated HSCs is crucial in preventing liver fibrosis. The role of activated HSCs and YKL-40 results, fascinating also, in the metastatic process in the liver, facilitating, probably, the homing of cancer cells. Despite several studies in this field, more investigations are needed to understand the functional role of this protein as target of cancer therapy. If YKL-40 has a role in the development of liver fibrosis, the inhibition of YKL-40 production or blocking of YKL-40 activity in patients with alcoholic liver disease or hepatitis C or B virus may be a valuable method for inhibiting the development of liver fibrosis.

Summary Points

1. YKL-40 (also known as chitinase 3-like 1) is a glycoprotein that belongs to this family.
2. Serum YKL-40 was significantly related to the degree of liver fibrosis with the highest levels in patients with moderate to severe fibrosis.
3. The highest serum YKL-40 levels were found in patients with metastatic tumor with poorest prognosis.
4. Potential molecules have been proposed to inhibit YKL-40 activity.
5. YKL-40 could be a helpful serum marker to estimate the degree of liver fibrosis.
6. Its inhibition could be expected to have therapeutic efficacy in liver disease.

References

Angulo P, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, Enders F, Saksena S, Burt AD, Bida JP, Lindor K, Sanderson SO, Lenzi M, Adams LA, Kench J, Therneau TM, Day CP. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology. 2007;45(4):846–54.

Berres ML, Papen S, Pauels K, Schmitz P, Zaldivar MM, Hellerbrand C, Mueller T, Berg T, Weiskirchen R, Trautwein C, Wasmuth HE. A functional variation in CHI3L1 is associated with severity of liver fibrosis and YKL-40 serum levels in chronic hepatitis C infection. J Hepatol. 2009;50(2):370–6.

Boot RG, van Achterberg TA, van Aken BE, Renkema GH, et al. Strong induction of members of the chitinase family of proteins in atherosclerosis: chitotriosidase and human cartilage gp-39 expressed in lesion macrophages. Arterioscler Thromb Vasc Biol. 1999;19:687–94.

Cho SJ, Echevarria GC, Lee YI, Kwon S, Park KY, Tsukji J, Rom WN, Prezant DJ, Nolan A, Weiden MD. YKL-40 is a protective biomarker for fatty liver in word trade center particulate matter- exposed firefighters. J Mol Biomark Diagn. 2014;5.

Chupp GL, Lee CG, Jarjour N, Shim YM, et al. A chitinase-like protein in the lung and circulation of patients with severe asthma. N Engl J Med. 2007;357:2016–27.

Cintin C, Johansen JS, Christensen IJ, Price PA, Sørensen S, Nielsen HJ. Serum YKL-40 and colorectal cancer. Br J Cancer. 1999;79:1494–9.

Cintin C, Johansen JS, Christensen IJ, Price PA, Sørensen S, Nielsen HJ. High serum YKL-40 level after surgery for colorectal carcinoma is related to short survival. Cancer. 2002;95:267–74.

Dehn H, Høgdall EV, Johansen JS, Jørgensen M, Price PA, Engelholm SA, Høgdall CK. Plasma YKL-40, as a prognostic tumor marker in recurrent ovarian cancer. Acta Obstet Gynecol Scand. 2003;82:287–93.

Faouzi S, Lepreux S, Bedin C, Dubuisson L, Balabaud C, Bioulac-Sage P, Desmouliere A, Rosenbaum J. Activation of cultured rat hepatic stellate cells by tumoral hepatocytes. Lab Investig. 1999;79:485–93.

Fontana RJ, Litman HJ, Dienstag JL, Bonkovsky HL, Su G, Sterling RK, Lok AS. HALT-C Trial Group. YKL-40 genetic polymorphism and the risk of liver disease progression in patients with advanced fibrosis due to chronic hepatitis C. Liver Int. 2012;32(4):665–74.

Hakala BE, White C, Recklies AD. Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. J Biol Chem. 1993;268:25803–10.

Hayes DF, Bast RC, Desch CE, Fritsche H, Kemeny NE, Jessup JM, Locker GY, Macdonald JS, Mennel RG, Norton L, Ravdin P, Taube S, Winn RJ. Tumor marker utility grading system: a framework to evaluate clinical utility of tumor markers. J Natl Cancer Inst. 1996;88:1456–66.

Hu B, Trinh K, Figueira WF, Price PA. Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. J Biol Chem. 1996;271:19415–20.

Jefri M, Huang YN, Huang WC, Tai CS, Chen WL. YKL-40 regulated epithelial–mesenchymal transition and migration/invasion enhancement in non-small cell lung cancer. BMC Cancer. 2015;15(1):590.

Jensen BV, Johansen JS, Price PA. High levels of serum HER-2/neu and YKL-40 independently reflect aggressiveness of metastatic breast cancer. Clin Cancer Res. 2003;9:4423–34.

Johansen JS. Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. Dan Med Bull. 2006;53:172–209.

Johansen JS, Cintin C, Jørgensen M, Kamby C, Price PA. Serum YKL-40: a new potential marker of prognosis and location of metastases of patients with recurrent breast cancer. Eur J Cancer. 1995;31A:1437–42.

Johansen JS, Møller S, Price PA, Bendtsen F, Junge J, Garbarsch C, Henriksen JH. Plasma YKL-40: a new potential marker of fibrosis in patients with alcoholic cirrhosis? Scand J Gastroenterol. 1997;32:582–90.

Johansen JS, Baslund B, Garbarsch C, Hansen M, et al. YKL-40 in giant cells and macrophages from patients with giant cell arteritis. Arthritis Rheum. 1999;42:2624–30.

Johansen JS, Christoffersen P, Møller S, Price PA, Henriksen JH, Garbarsch C, Bendtsen F. Serum YKL-40 is increased in patients with hepatic fibrosis. J Hepatol. 2000;32:911–20.

Junker N, Johansen JS, Andersen CB, Kristjansen PE. Expression of YKL-40 by peritumoral macrophages in human small cell lung cancer. Lung Cancer. 2005;48:223–31.

Kamal SM, Turner B, He Q, Rasenack J, et al. Progression of fibrosis in hepatitis C with and without schistosomiasis: correlation with serum markers of fibrosis. Hepatology. 2006;43:771–9.

Lebensztejn DM, Wierzbicka A, Socha P, Pronicki M, Skiba E, Werpachowska I, Kaczmarski M. Cytokeratin-18 and Hyaluronic acid level predict liver fibrosis in children with non-alcoholic fatty liver disease. Acta Biochim Pol. 2011;58(4):563–6.

Lunevicius R, Nakanishi H, Ito S, Kozaki K-I, Kato T, Tatematsu M, Yasui K. Clinicopathological significance of fibrotic capsule formation around liver metastasis form colorectal cancer. J Cancer Res Clin Oncol. 2001;127:193–9.

Neaud V, Faouzi S, Guirouilh J, Le Bail B, Balabaud C, Bioulac-Sage P, Rosenbaum J. Human hepatic myofibroblasts increase invasiveness of hepatocellular carcinoma cells: evidence for hepatocyte growth factor. Hepatology. 1997;26(6):1458–66.

Nøjgaard C, Johansen JS, Christensen E, Skovgaard LT, Price PA, Becker U, The EMALD Group. Serum levels of YKL-40 and PIIINP as prognostic markers in patients with alcoholic liver disease. J Hepatol. 2003a;39:179–86.

Nøjgaard C, Johansen JS, Krarup HB, Holten-Andersen M, Møller A, Bendtsen F, Danish Viral Hepatitis Study Group. Effect of antiviral therapy on markers of fibrogenesis in patients with chronic hepatitis. Scand J Gastroenterol. 2003b;38:659–65.

Olaso E, Santisteban A, Bidaurrazaga J, Gressner AM, Rosenbaum J, Vidal-Vanaclocha F. Tumor-dependent activation of rodent hepatic stellate cells during experimental melanoma metastasis. Hepatology. 1997;26:634–42. 32.

Pinzani M. Liver fibrosis. Springer Semin Immunopathol. 1999;21:475–90.

Recklies AD, White C, Ling H. The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. Biochem J. 2002;365(Pt 1):119–26.

Rehli M, Krause SW, Andreesen R. Molecular characterization of the gene for human cartilage gp-39 (CHI3L1), a member of the chitinase protein family and marker for late stages of macrophage differentiation. Genomics. 1997;43:221–5.

Rehli M, Niller H-H, Ammon C, et al. Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation. J Biol Chem. 2003;278:44058–67.

Saitou Y, Shiraki K, Yamanaka Y, Yamaguchi Y, Kawakita T, Yamamoto N, Sugimoto K, Murata K, Nakano T. Non invasive estimation of liver fibrosis and response to interferon therapy by a serum fibrogenesis marker, YKL-40, in patients with HCV-associated liver disease. World J Gastroenterol. 2005;11(4):476–81.

Shackel NA, McGuinness PH, Abbott CA, Gorrell MD, McCaughan GW. Novel differential gene expression in human cirrhosis detected by suppression subtractive hybridization. Hepatology. 2003;38:577–88.

Shimizu S, Yamada N, Sawada T, Ikeda K, Kawada N, Seki S, Kanede K, Hirakawa K. In vivo and in vitro interactions between human colon carcinoma cells and hepatic stellate cells. Jpn J Cancer Res. 2000;91:1285–95.

Smith JA. Neutrophils, host defense, and inflammation: a double-edged sword. J Leukoc Biol. 1994;56:672–86.

Tao H, Yang JJ, Shi KH, Huang C, Zhang L, Lu XW, Li J. The significance of YKL 40 protein in liver fibrosis. Inflamm Res. 2014;63(4):249–54.

Tarpaag LS, Guren TK, Glimelius B, Christensen IJ, Pfeiffer P, Kure EH, Sorbye H, Ikdahl T, Yilmaz M, Johansen JS, Tveit KM. Plasma YKL-40 in patients with metastatic colorectal cancer treated with first line oxaliplatin- based regimen with or without cetuximab: RESULT from the NORDIC VII Study. PLoS One. 2014;9(2):e87746.

Tran A, Benzaken S, Saint-Paul M-C, Guzman-Granier E, Hastier P, Pradier C, Barjoan EM, Demuth N, Longo F, Rampal P. Chondrex (YKL-40), a potential new serum fibrosis marker in patients with alcoholic liver disease. Eur J Gastroenterol Hepatol. 2000;12:989–93.

Volck B, Johansen JS, Stoltenberg M, Garbarsch C, et al. Studies on YKL-40 in knee joints of patients with rheumatoid arthritis and osteoarthritis. Involvement of YKL-40 in the joint pathology. Osteoarthritis Cartilage. 2001;9:203–14.

Phosphatidylethanol and Alcohol Use in Liver Disease Patients

25

Scott H. Stewart

Contents

Abstract

Alcohol use can potentiate liver diseases such as hepatitis C and in sufficient quantities can itself cause significant liver disease over time. This chapter will briefly review the importance of alcohol use in liver diseases and difficulties

S.H. Stewart (✉)
Department of Medicine, University at Buffalo, State University of New York, Buffalo, NY, USA
e-mail: ss243@buffalo.edu

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_18

that can arise when clinicians attempt to gather an accurate drinking history in patients with liver disease. The bulk of the chapter focuses on the use of blood phosphatidylethanol (PEth) as an alcohol consumption biomarker in liver disease patients, including validation in this population, potential uses in clinical care and research, and current limitations to its use for these purposes.

Keywords

Alcohol drinking • Liver disease • Phosphatidylethanol • Sensitivity • Specificity

List of Abbreviations

%dCDT	% disialo-carbohydrate-deficient transferrin
ALT	Alanine transaminase
AST	Aspartate aminotransferase
CDT	Carbohydrate-deficient transferrin
ELSD	Evaporative light scattering detection
EtG	Ethyl glucuronide
GGT	Gamma-glutamyltransferase
HPLC	High-performance liquid chromatography
MCV	Mean corpuscular volume
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PEth	Phosphatidylethanol
ROC	Receiver operating characteristic

Key Facts if PEth Is Used as a Test for Drinking in Liver Disease Patients

- Detection of phosphatidylethanol (PEth) in blood means an individual has consumed alcohol within the past several weeks and in theory extends up to roughly 2 months if drinking had been heavy.
- If PEth is not detected in blood, an individual may still be drinking, but generally not enough to harm their liver or worsen the effects of liver conditions such as hepatitis C.
- Generally speaking, the higher the PEth, the more alcohol has been consumed in the prior days and weeks, but people vary in how much PEth they make in response to a particular degree of drinking.
- The concentration of PEth can be used to predict alcohol use that is heavy or may harm the liver, but some individuals will be falsely labeled as relatively heavy drinkers, and some heavy drinkers will be missed, if PEth is used in this manner.

Definitions of Words and Terms

Carbohydrate-deficient transferrin (or disialo-carbohydrate-deficient transferrin)	A test for heavy alcohol use that results from a heavy drinking-induced decrease in transferrin glycosylation.
Direct alcohol consumption biomarker	A laboratory test for a product resulting from the metabolism of ethanol, as opposed to an indirect test that usually involves products resulting from alcohol toxicity.
Ethyl glucuronide (EtG)	A product of ethanol metabolism that can be used to estimate recent alcohol use. It is often measured in the urine or hair.
Evaporative light scattering detection (ELSD)	A method for quantifying a substance based on dispersion of light in a vaporized sample.
High-performance liquid chromatography (HPLC)	A method for separating a sample into smaller fractions based on differences in physical characteristics such as molecular mass.
Mass spectrometry (MS)	A method used to separate a complex sample into its various constituents (e.g., isolate a specific phosphatidylethanol from other phosphatidylethanols).
Phosphatidylethanol (PEth)	A phospholipid formed only in the presence of ethanol. It can be measured in the red blood cell membrane as a test for alcohol consumption.
Receiver operating characteristic (ROC) analysis	A method for estimating the ability of a test to accurately identify the presence or absence of a particular condition.
Sensitivity	The probability that a test for a particular condition will be positive when that condition is truly present.
Specificity	The probability that a test for a particular condition will be negative when that condition is truly absent.
Tandem mass spectrometry (MS/MS)	A method for confirming the identity of a specific molecule isolated by MS and can be used to quantify that molecule relative to the known concentration of a standard added to the sample. It is often paired with an initial HPLC step.

Introduction

Alcohol and Liver Disease

Chronic, heavy alcohol consumption is a major cause of serious liver disease, which generally proceeds through an initial and reversible fatty liver disease phase, to chronic hepatitis and progressive fibrosis, and eventually to cirrhosis if drinking continues (Cohen and Nagy 2011). In epidemiologic studies, the incidence of alcoholic liver disease begins to increase after an average daily alcohol intake of approximately 30 g, but a much greater risk is associated with years of heavier use in the neighborhood of 100 g or more daily (Zakhari and Li 2007). Equal in importance to primary alcoholic liver disease, alcohol use can contribute to the progression of other liver disease etiologies such as chronic hepatitis C infection (Szabo et al. 2010). In the USA, about one-half of liver disease deaths are due to alcohol (Yoon and Yi 2010), including roughly 70% of deaths occurring in patients under the age of 35. Alcoholic liver disease alone accounted for 18.4% of all liver transplants in 2013 and was the primary diagnosis in 24.1% of patients awaiting transplantation (Kim et al. 2015). The contribution of alcohol use to the progression of other liver diseases is difficult to quantify but is substantial. For example, alcoholic liver disease has been estimated to cause one-third of incident hepatocellular cancers (Barve et al. 2008). Clearly, accurate estimates of alcohol exposure are a critical component of the clinical assessment and care for patients with liver conditions, but this can be a difficult task using traditional methods. This chapter focuses on a newer alcohol consumption biomarker, blood phosphatidylethanol, which has shown promise as an objective test for alcohol use.

Limitations of Alcohol Use Estimates Obtained by Patient-Reporting and Traditional Biomarkers of Alcohol Consumption

Alcohol consumption that is sufficient to cause liver disease or exacerbate other chronic liver conditions is clinically important to detect in order to limit unnecessary diagnostic testing, direct treatment, and monitor treatment response. However, a number of factors can prevent detection of potentially harmful drinking (Del Boca and Darkes 2003). Healthcare providers may take inaccurate or insufficient drinking histories, which requires at least knowledge of beverages consumed and their approximate alcohol content, how often drinking occurs, and what amount of alcohol is consumed on typical drinking days and on heavier drinking days. This can also be estimated using a standardized screening instrument such as the full ten-item Alcohol Use Disorders Identification Test or shorter variants (Babor et al. 1992; Bradley et al. 2007). However, even standardized and validated self-report screening will not overcome underreporting due to cognitive dysfunction (e.g., frontal lobe dysfunction resulting from years of heavy drinking or hepatic encephalopathy) or intentional underreporting to avoid potential penalties or avoid discussion of a stigmatizing topic. As an example, 18 alcoholic liver disease patients

awaiting transplantation in Germany were asked to provide information on their alcohol use. In addition to patient self-report, urine ethyl glucuronide was measured, which above a minimal cutoff to eliminate unintentional alcohol exposure is highly specific for drinking within the past 3 or 4 days, including in cirrhotic patients (Staufer et al. 2011). All of the patients reported abstinence, but half were found to have positive assays for urine ethyl glucuronide (Erim et al. 2007). As the authors concluded, underreporting of alcohol use and false reporting of abstinence may be particularly important in patients with cirrhosis, as continued drinking may harm their candidacy for liver transplantation. On the other end of the spectrum, patients may be harmed if they are truly abstinent but are clinically suspected to be drinking due to prior health histories. In either case, in order to direct care and optimize outcomes for individual patients, it is crucial to obtain objective and accurate information on alcohol use.

Laboratory testing for alcohol use can supplement self-report, but there is currently no highly valid (i.e., few falsely positive or falsely negative results) and widely available test for detecting potentially problematic alcohol use. Detection of ethanol itself in the blood, breath, or urine is highly specific and inexpensive, but ethanol elimination from blood proceeds at roughly the equivalent of one drink per hour, and any un-metabolized ethanol is rapidly excreted from urine. Thus the sensitivity of this form of screening or monitoring is limited to hours following drinking cessation, and low concentrations do not reveal the magnitude of even very recent drinking. Traditional heavy alcohol consumption biomarkers include markers of liver injury such as the transaminases, aspartate aminotransferase (AST) and alanine transaminase (ALT), and gamma-glutamyltransferase (GGT). Other heavy drinking biomarkers include erythrocyte mean corpuscular volume (MCV) and carbohydrate-deficient transferrin (CDT). All of these can be considered as "toxic effect" biomarkers (e.g., end organ effects of heavy drinking). All such markers are useful only for modestly shifting the probability of chronic heavy drinking and have limited sensitivity even for that pattern of drinking (Conigrave et al. 2003). Furthermore, specificity is even lower in clinical populations where other causes of laboratory abnormalities are most prevalent. Particularly relevant to the detection of heavy drinking, liver disease itself often results in abnormalities in AST, ALT, GGT, CDT, and erythrocyte MCV.

Direct Biomarkers of Alcohol Consumption

Ingested alcohol is overwhelmingly metabolized to the intermediate acetaldehyde by alcohol dehydrogenase, with an additional important oxidative pathway that includes the inducible cytochrome P450 system. However, there are numerous alternate pathways for alcohol elimination that account for a minor portion of ingested ethanol and produce a number of products that can be measured in various matrices such as blood, hair, and urine. These are not toxic effect markers, and thus their formation does not require end organ effects of heavy drinking, and, since alcohol is required for their synthesis, other conditions such as liver disease will not

result in their production. Thus they offer the potential to detect alcohol use with highly objective testing as a supplement to or in lieu of patient self-reporting, even in a clinically heterogeneous population. Due to their specificity for some degree of alcohol exposure, there has been a slowly progressing development of some of these non-oxidative minor elimination products as an alternate class of alcohol consumption biomarkers. The most studied include the aforementioned ethyl glucuronide in hair and urine and phosphatidylethanol in blood. As the name implies, ethyl glucuronide is the result of ethanol elimination via glucuronidation. This is a highly water-soluble product that is rapidly eliminated from blood (extending a few hours past the elimination of ethanol itself), but can be detected in urine for roughly 1–4 days. Ethyl glucuronide also accumulates in hair, where it can be detected for months following the cessation of moderate to heavy drinking. Ethyl glucuronide in both urine and hair is a valid test for drinking in patients with liver diseases (Stewart et al. 2013a, b), but this chapter is focused on phosphatidylethanol (PEth) for reasons highlighted throughout the remainder of this chapter, including a half-life that is clinically useful and the common use of blood testing in medical settings.

PEth is a phospholipid formed only in the presence of ethanol and was discovered in vivo by two research groups in 1983 (Wrighton et al. 1983; Alling et al. 1983). It results from a phospholipase D-catalyzed transphosphatidylation reaction in cell membranes and is a product of ethanol and native membrane phospholipids (Gustavsson 1995). A similar reaction is used in research laboratories to detect and quantify phospholipase D activity. PEth is actually a family of phospholipids due to the variety of native phospholipids with which ethanol can react, and numerous PEths are thus detectable in blood drawn from heavy alcohol consumers (Gnann et al. 2010). Each unique PEth consists of a two-carbon chain from ethanol with a glycerophosphate backbone and two fatty acid side chains, with the specific species being determined by the fatty acid components. The dominant native phospholipid is phosphatidylcholine, and the reaction between abundant forms of phosphatidylcholine and ethanol produces the most abundant PEth species in humans, namely, the isomers 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanol and 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphatidylethanol (16:0/18:1). These species account for approximately 35–40% of total blood PEth (Gunnarsson et al. 1998; Holbrook et al. 1992). PEth will accumulate in erythrocytes if alcohol is consumed and can be measured following disruption of the erythrocyte membrane and extraction of lipids with organic solvents.

Two main assays have been developed for quantifying PEth from whole blood, the bulk of which is in the erythrocyte membrane (Varga et al. 2000). Separation of the PEth fraction of membrane lipids by high-performance liquid chromatography (HPLC) followed by quantification by evaporative light scattering detection (ELSD) was a relatively early assay (Gunnarsson et al. 1998) and was used in a number of European studies that initially evaluated PEth as an alcohol consumption biomarker (Aradottir et al. 2006; Hartmann et al. 2007; Wurst et al. 2004a). This assay detects a family of PEths rather than a specific species. Human studies using this assay method or thin-layer chromatography suggested that detectable PEth was highly sensitive and specific for heavy drinking (Aradottir et al. 2006; Hansson et al. 1997;

Hartmann et al. 2007; Wurst et al. 2004b). Mass spectrometry (MS)-based assays with low limits of detection and quantification have also been developed. These assays again use HPLC separation followed by detection using tandem MS (HPLC-MS/MS), with the 16:0/18:1 isomers being quantified relative to a known concentration of labeled PEth or another phosphatidylalcohol. These assays have generally revealed quantifiable PEth in light to moderate drinkers as well as heavy drinkers (e.g., Stewart et al. 2010; Hahn et al. 2012; Kwak et al. 2012; Jain et al. 2014), with a moderately strong correlation with total alcohol consumption in the prior weeks ($r \sim 0.6$). PEth undergoes nonspecific degradation with a half-life of roughly 5–10 days (Varga et al. 2000; Gnann et al. 2012). This correlation with total consumption and half-life is ideal for monitoring response to treatment for problem drinking, as a substantial drop in PEth would occur within 2 weeks of successful treatment initiation, whereas minimal change would suggest a continuation of a similar magnitude of drinking. Given that PEth requires ethanol for synthesis, that blood is easily and routinely obtained in clinical settings, and that PEth will persist for many days following drinking cessation, there is clear potential for clinical utility that is far superior to the detection of ethanol itself or measurement of traditional toxic effect biomarkers.

PEth in Patients with Liver Disease

In clinical settings, an ideal use of highly specific alcohol biomarkers might include application in conditions frequently caused or exacerbated by alcohol and where self-report may be less reliable or in some cases unobtainable due to severe illness. Liver disease fits these criteria due to the common diagnosis of alcoholic liver disease, the severity of liver diseases which can result in encephalopathy or even coma, and potential incentives to underreport drinking such as liver transplantation eligibility or avoidance of stigma. However, detection of problematic alcohol use is important in order to direct treatment with the goal of achieving optimal health outcomes for patients.

Early studies on PEth in non-liver disease subjects using HPLC-ELSD assays suggested extremely high sensitivity and specificity for heavy drinking when PEth was detectable, but often did not include intermediate-level drinkers who may be consuming alcohol at safe or even elevated levels short of truly heavy consumption (e.g., less than 60 g daily). The development of HPLC-MS/MS assays provided a means for detecting and quantifying very low concentrations of PEth (limit of quantitation now as low as 8 ng/mL and limits of detection as low as 2 ng/mL) and increased the likelihood that PEth could be detected in even light to moderate drinkers, with cutoff concentrations that might be useful for differentiating abstainers or very light drinkers from moderate drinkers and in turn moderate drinkers from heavy drinkers.

In a proof of concept study (Stewart et al. 2009), 42 subjects were recruited from a clinical setting, including 21 with liver disease and 21 with hypertension (another potentially alcohol-related condition). Alcohol use was estimated using a timeline

followback interview that is often used in epidemiologic studies on alcohol use (Sobell and Sobell 1992). The technique involves patient self-recall of daily alcohol use that takes advantage of a calendar including events that are memorable for the patient as modifiers of their usual drinking patterns. In the liver disease subjects, 12 reported consuming alcohol in the prior month, with average consumption ranging from <1 to 178 g of ethanol daily in the prior 2 weeks. PEth was measured by a contracted laboratory using an HPLC-MS/MS assay with a limit of quantitation of 20 ng/mL. In this group, 11 had quantifiable PEth, which included four out of five subjects averaging less than two drinks daily. The only drinker without detectable PEth reported an average of 1 g daily (i.e., had consumed one standard US drink in the prior 2 weeks). Thus PEth was clearly quantifiable in light to moderate drinkers with liver disease.

A subsequent larger study was undertaken to define the association between PEth and alcohol use, determine its ability to differentiate drinking and various degrees of drinking from abstinence and lighter alcohol use, and compare PEth to % disialo-carbohydrate-deficient transferrin (%dCDT) in detecting heavy drinking. The performance of PEth for detecting heavy drinking (averaging ≥56 g of ethanol daily) was reported (Stewart et al. 2014). Those findings are summarized here and supplemented with additional findings for detecting moderate drinking and after eliminating a small number of subjects with inaccurate reporting of alcohol use. %dCDT is a well-characterized biomarker of heavy drinking (≥60 g daily). At the commonly used cutoff concentration, %dCDT is approximately 60% sensitive and 95% specific (Bergstrom and Helander 2008), but performance suffers in advanced liver disease due to abnormal transferrin glycovariants (Arndt et al. 2008; DiMartini et al. 2001). Subjects included 222 liver disease patients who were recruited at the time of an outpatient visit with a hepatologist or during a hospitalization for liver disease. Alcohol use during the preceding 90 days was again estimated using the timeline followback method; blood and other samples were obtained for laboratory testing, and the electronic medical record was reviewed to determine liver disease etiology and severity. In order to have as accurate an account of alcohol use as possible for PEth validation, subjects' hair and urine were also assayed for ethyl glucuronide (EtG) using HPLC-MS/MS assays (Morini et al. 2006; Dresen et al. 2004). In the urine, EtG may persist for 4 or 5 days following drinking cessation (Wurst et al. 2002), and EtG may be detectable in hair for several months following the cessation of heavy drinking (Pragst et al. 2010). Thus, subjects who reported at least 7 days of abstinence but had urine EtG >100 ng/mL and subjects reporting at least 90 days of abstinence but having hair EtG ≥30 pg/mg were excluded. These were conservative criteria, as urine EtG would not normally persist as long as 1 week, and that concentration of EtG in hair is strongly suggestive of heavy drinking, rather than any drinking or even light to moderate drinking (Society of Hair Testing 2014). Excluding subjects with mismatches between these biomarkers and alcohol self-report resulted in the elimination of 12 out of 222 subjects (5.4%). Characteristics of these 210 study participants are listed in Table 1.

PEth was again measured by an HPLC-MS/MS assay at a contracted laboratory that was blinded to the alcohol and clinical data. Receiver operating characteristic

Table 1 Sample characteristics stratified by past-month alcohol use

Characteristic	Abstinent ($n = 90$)	<28 g daily ($n = 60$)	28 to <56 g daily ($n = 25$)	≥56 g daily ($n = 35$)
Age (SD)	53 (10)	50 (13)	48 (12)	52 (8)
Gender (% male)	51	48	72	71
Alcohol-related disease (%)	21	17	64	86
Quantifiable PEth (%)	2	55	96	100
Median grams of alcohol per day (IQR)	—	7 (2–17)	41 (34–45)	124 (81–162)

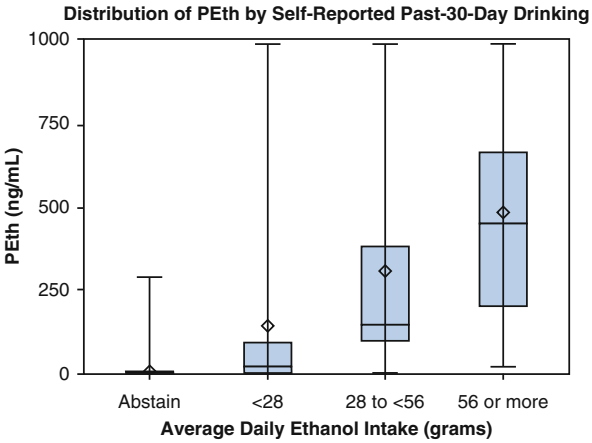

Fig. 1 Ends of *boxes* represent the 25th and 75th percentiles, *lines* within *boxes* represent the median, *diamonds* represent means, and *whiskers* represent the range (capped at 1,000 ng/mL for illustration)

(ROC) analyses were completed to estimate the ability of PEth to differentiate various levels of alcohol consumption, and stratified analyses were undertaken to evaluate differences in the PEth-alcohol relationship attributable to factors such as liver disease severity.

The relationship between PEth and alcohol use is illustrated in Fig. 1, with a steady increase in median PEth concentrations as alcohol use increased, but with substantial overlap. Results of ROC analyses are shown in Fig. 2. The panels show that PEth is best at differentiating moderate to heavy alcohol use from lighter alcohol use and abstinence, but relatively less useful for differentiating any drinking from abstinence. These daily averages were selected as a standard drink in the USA, where the study was completed, containing 14 g of ethanol. In addition, PEth was superior to %dCDT for differentiating heavy drinking from lighter drinking and abstinence (area under the curve 0.907 vs. 0.792, $p = 0.026$). The curves demonstrate that PEth does not strongly differentiate "any drinking" from abstinence, mainly because many lighter drinkers did not have detectable PEth. Its performance in differentiating liver patients reporting at least 28 g daily or at least 56 g daily was similar, with areas under the curve of approximately 90%.

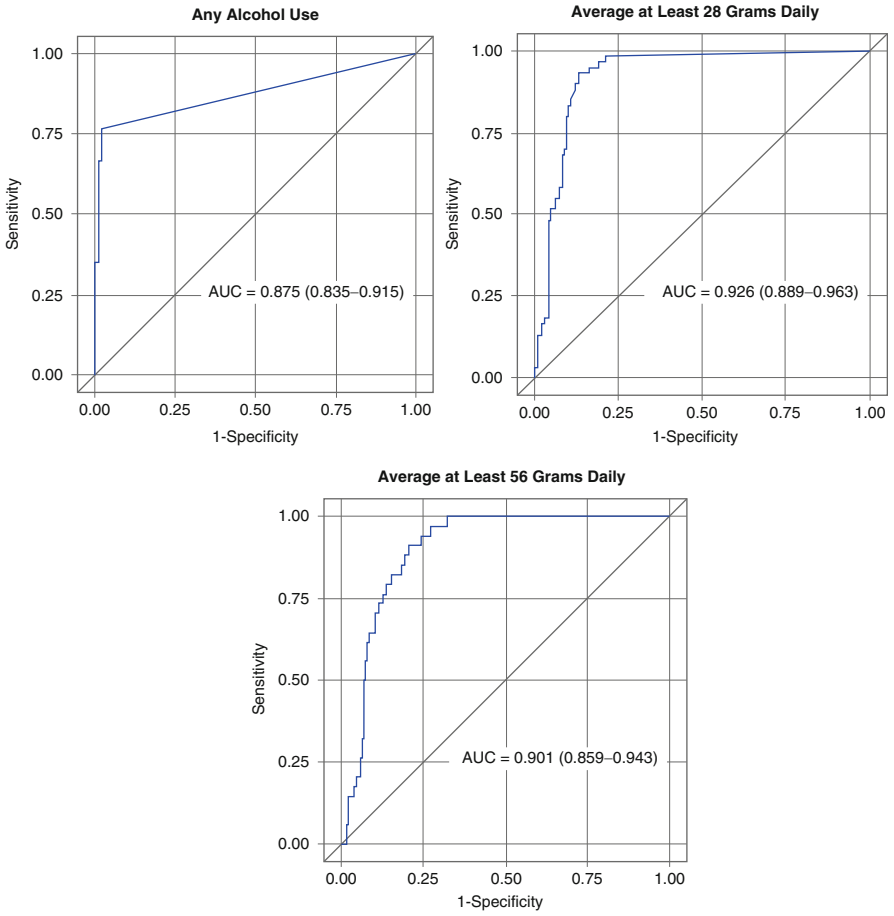

Fig. 2 ROC curves for detecting various drinking levels and receiver operating characteristic curves for PEth detection of various levels of average daily alcohol use. An area under the curve (*AUC*) of 0.90, for example, indicates that PEth would be higher in individuals drinking above the indicated limit compared to a person drinking under the limit 90% of the time

One interpretation of the area under the ROC curve would be that PEth would be higher in subjects drinking at least 28 (or 56) grams relative to lighter drinkers and abstainers in nine of ten randomly selected pairs from the study population that included one subject from each group. In clinical settings, the sensitivity (i.e., the probability of a test being positive in the presence of a particular condition) and specificity (i.e., the probability of a test being negative in the absence of a particular condition) are often estimated as measures of test performance. This requires selecting a cutoff concentration, with higher cutoffs favoring specificity to lower the probability of falsely positive test results and lower cutoffs favoring sensitivity to lower the probability of falsely negative results. One means of "trading off" sensitivity and specificity is by selecting a cutoff concentration that maximizes the

Youden index, which is the value of sensitivity + specificity – 1. This identifies the point on the ROC curve that is the most distant from the diagonal (or the most distant from the performance of a useless test). Another way would be to select a minimally acceptable specificity and calculate sensitivity at the highly specific cutoff concentration. High specificity may be particularly valuable when screening for problematic alcohol use in order to minimize false labeling of a patient as a heavy drinker. Using these methods, selections of potential cutoff concentrations for PEth in detecting moderate to heavier drinking are listed in Table 2. The Youden index provides a reasonable cutoff concentration for detecting an average of at least 28 g daily, with loss of sensitivity for little gain in specificity at higher cutoffs. For detecting heavier drinking (in this case averaging at least 56 g daily), a more specific cutoff may be desirable to minimize falsely labeling patients.

The limit of quantitation provides the optimal cutoff for detecting any alcohol use. At the initial quantitation limit (20 ng/mL), PEth sensitivity was 0.70 (0.60–0.79), and specificity was 0.97 (0.93–1.00) for drinking in the prior 30 days. During the course of the study, the limit of quantitation was lowered due to improved instrumentation. In the 126 subjects for whom the lower quantitation limit (8 ng/mL) was available, PEth sensitivity was 0.79 (0.71–0.88) and specificity was 0.90 (0.81–0.98).

Liver disease can range from mild (e.g., fatty infiltration or minor inflammation) to severe (e.g., advanced fibrosis and cirrhosis). Thus, in order to inform optimal use of the test, it is important to evaluate any effects of liver disease severity on the performance of PEth as an alcohol consumption biomarker. In this study, patients were considered to be cirrhotic if they had stigmata of portal hypertension (upper gastrointestinal tract varices or chronic ascites) or had biopsy-proven cirrhosis. Using these criteria, 110 subjects were considered cirrhotic (52%) and 100 were considered not cirrhotic (48%). Of the 110 cirrhotic subjects, 34 (31%) had quantifiable PEth, and of the 100 non-cirrhotic subjects, 60 (60%) had quantifiable PEth. In these subjects, the effect of cirrhosis on the PEth-alcohol relationship was evaluated by regressing log PEth on past-30-day alcohol use, cirrhosis, and their interaction. This suggested that liver disease severity (i.e., comparing cirrhotic to non-cirrhotic subjects) did not change the effect of alcohol use on PEth concentration (p-value for interaction term = 0.399). Although the interaction was not significant, the sample size was relatively small, and the relationship between PEth and alcohol use could still differ quantitatively between subjects with cirrhotic and non-cirrhotic liver disease, as illustrated in Fig. 3. Graphically, these results do not rule out a more positive relationship in non-cirrhotic subjects, and this is an area in need of continued research.

Why Did Some Subjects Reporting at Least 1-Month Abstinence Have Quantifiable PEth?

In this study, there were two subjects with quantifiable PEth who reported abstinence during the prior 30 days. Both reported heavy drinking that stopped 5 and 6 weeks

Table 2 Sensitivity and specificity of PEth for various levels of drinking

	Cutoff selection method					
	Maximal Youden index			Higher specificity		
	Concentration	Sensitivity	Specificity	Concentration	Sensitivity	Specificity
Average at least 28 g	52 ng/mL	0.93	0.87	100 ng/mL	0.82	0.90
		(0.87–1.00)	(0.81–0.92)		(0.72–0.91)	(0.85–0.95)
Average at least 56 g	89 ng/mL	0.91	0.80	295 ng/mL	0.75	0.91
		(0.82–1.00)	(0.74–0.86)		(0.58–0.92)	(0.86–0.96)

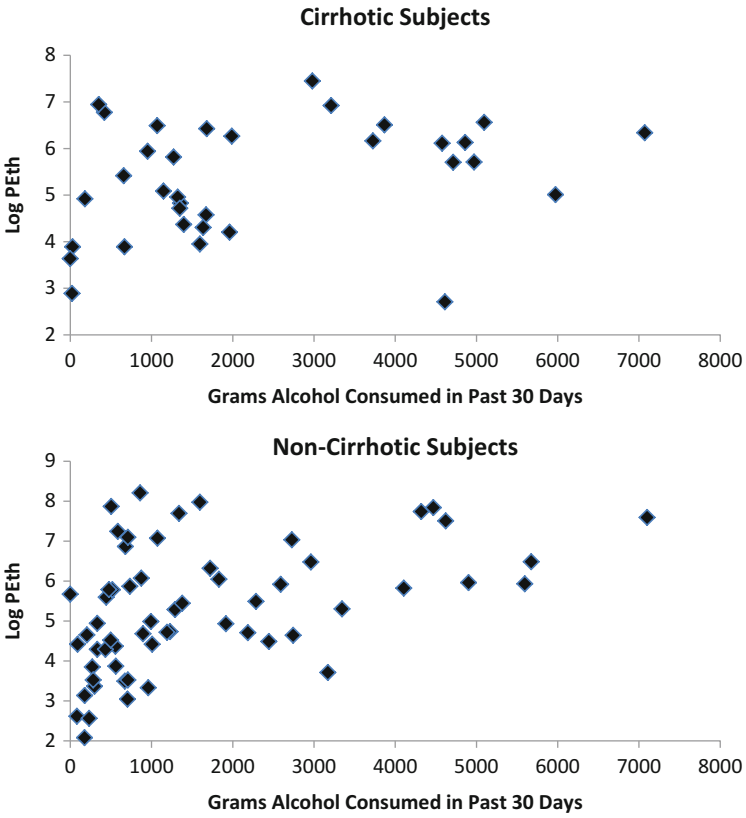

Fig. 3 Relationship between PEth (log transformed for normality) and past-month alcohol consumption (capped at 7,000 g for illustration) in cirrhotic and non-cirrhotic subjects with quantifiable PEth

prior to PEth measurement. The elimination of PEth from erythrocytes has not been completely characterized, but a conservative estimate of the PEth half-life is roughly 10 days. Assuming that elimination does not depend on concentration, on average, individuals with a PEth concentration of about 175 ng/mL or higher at the time of drinking cessation might have quantifiable PEth for more than 30 days. This would include a substantial proportion of heavy drinkers, as has been shown previously for alcohol detoxification patients (Wurst et al. 2010). Thus PEth was likely still being eliminated from the two 1-month abstainers with quantifiable PEth in this study.

Who Were the Past-Month Drinkers Without Quantifiable PEth?

A large majority of moderate to heavy drinkers will develop a quantifiable PEth concentration. All of the subjects reporting an average daily alcohol intake of 56 g or

more had quantifiable PEth, and 59/60 (98%) subjects consuming an average of at least 28 g had quantifiable PEth. One subject in this group without quantifiable PEth reported abstinence for 25 days prior to PEth measurement, but prior heavy drinking was sufficient to result in an average daily alcohol intake of 40 g during the preceding month. In drinkers averaging less than 28 g daily, PEth was quantifiable in 33/60 (55%). Within this lighter drinking group, the 27 subjects without quantifiable PEth reported a median of 2.1 g/daily, and the 33 subjects with quantifiable PEth reported a median of 14.4 g/daily ($p < 0.001$). Thus consuming roughly one drink daily or more usually resulted in quantifiable PEth.

Potential Applications of PEth for Detection and Monitoring of Alcohol-Related Liver Diseases

Advantages That May Be Realized from the Use of PEth in the Care of Patients with Liver Disease and Clinical Research on Alcohol-Related Liver Diseases

The introductory sections of this chapter outlined the importance of alcohol use in liver disease etiology and outcomes and difficulties that arise in quantifying alcohol use, particularly in patients with liver disease. The use of blood PEth has a number of potential benefits in research and clinical care. For observational research, the contribution of alcohol use to the progression of other common liver conditions (e.g., hepatitis C infection, nonalcoholic fatty liver disease, etc.) could be clarified with the use of objective biomarkers. In clinical trials, the role of alcohol, either as an important comorbidity for adjustment or screening purposes or a primary treatment outcome, could limit misclassification of subjects, allowing for more accurate results and efficient study designs. Clinically, PEth may aid in initial diagnostic evaluations for liver disease and provide an objective marker of treatment response. This has analogies to the use of glycosylated hemoglobin in diabetes care, where queries are made about glucose self-monitoring results, but treatment decisions are often made based on biomarker results. As shown in Fig. 1, PEth concentration does correlate with past-month alcohol use, albeit imperfectly. Thus, as in Table 1, specific cutoffs can be selected for various levels of average daily alcohol consumption, but some misclassification will occur. Monitoring of PEth remains an understudied tool, but the optimal clinical use of this biomarker will likely be the change in PEth occurring within an individual patient over time. The half-life of PEth will be particularly useful in this regard, as changes should be evident in the weeks following successful treatment or early in the course of relapse. PEth could also be incorporated into transplant candidacy evaluations where abstinence is required, although the best use of alcohol consumption biomarkers is clearly to improve outcomes for patients who have not been able to control their drinking, rather than as a punitive measure.

Known or Potential Limitations of PEth as an Alcohol Consumption Biomarker in Liver Disease Patients

As with any clinical test, PEth is far from perfect. No alcohol consumption biomarker will be better than an honest self-report from a patient with an intact memory, and this should remain the "gold standard" of alcohol assessment. However, in the absence of self-reported heavy drinking, it is often difficult to know when this gold standard has been achieved. Objective alcohol consumption biomarkers will thus have an important role in alcohol assessment. So, regarding PEth, it is important to note its substantial limitations.

Although a positive assay for PEth is specific for some degree of non-incidental alcohol exposure, it is not a test for abstinence. Many lighter drinkers will not have quantifiable PEth, even down to limits of quantitation of 8 ng/mL. However, the vast majority of liver disease patients who are consuming enough alcohol to harm their liver will have quantifiable PEth in their blood.

If PEth is not a test for abstinence, then is it a test for drinking? For the most part, the answer is yes. However, unintentional alcohol use could in theory result in the detection of PEth, particularly as the sensitivity of mass spectrometry assays increases over time. Also, non-beverage exposure (e.g., inhaled ethanol with vaping) can approach or even exceed beverage consumption and will circumvent first-pass metabolism in the liver. The resulting blood alcohol concentration would likely yield quantifiable PEth.

Interpretation of PEth is limited by the very reason it is clinically relevant. That is, imperfect knowledge of alcohol use, including average drinking, heavy drinking episodes with and without food, and when drinking or heavy drinking, may have ceased. All of these factors likely influence PEth concentration at any given point in time. However, this is not unlike other clinically useful biomarkers such as glycosylated hemoglobin, where interpretation assumes stable diabetes over months, but fluctuations in glucose control are likely. Interpretation of PEth concentrations would greatly benefit from research that is analogous to the linking of glycosylated hemoglobin levels to diabetes-relevant outcomes over time (UKPDS 1998). For example, the trajectory of PEth over time could be evaluated as a predictor of liver disease progression or recovery.

Differences in alcohol use patterns and resultant blood alcohol levels will account for some of the differences in PEth seen between subjects consuming a similar amount of alcohol on average. However, a major limitation in interpreting PEth assay results lies in the continued limited knowledge of individual differences in PEth synthesis and elimination. For example, some research has indicated that a prolonged history of heavy drinking, as would be the case in alcohol-dependent individuals, may result in more rapid PEth synthesis (Varga and Alling 2002; Mueller et al. 1988). This type of knowledge will accumulate over the years and may eventually increase the accuracy of interpretation, but for the present, this variability will contribute to misclassification of drinking status when a cutoff concentration is used.

Finally, PEth assays are expensive and not available in the large majority of hospital-affiliated clinical laboratories. To reach its full potential, automated and cost-effective assays will need to be developed, which due to low antigenicity is a more difficult task for a lipid-based biomarker compared to a protein biomarker (Nissinen et al. 2008).

Summary Points

- Alcohol use causes liver disease or potentiates other causes of liver disease.
- It is important to obtain accurate drinking data to optimize medical care.
- Drinking histories can be inaccurate due to poor technique, cognitive dysfunction, or intentional underreporting.
- Traditional laboratory tests for alcohol use are particularly inaccurate in patients with liver diseases.
- Non-oxidative alcohol elimination products such as blood phosphatidylethanol (PEth) are valid biomarkers of alcohol consumption in patients with liver disease.
- Quantifiable PEth is specific for some alcohol use in the prior weeks, but the absence of quantifiable PEth does not rule out light drinking.
- PEth concentration increases as drinking increases, and cutoffs can be used to identify certain degrees of alcohol use that may be sufficient to harm the liver either alone or in concert with other conditions such as hepatitis C infection.
- Additional research is needed to define individual differences in drinking patterns or other effect modifiers (e.g., genetic variation) of the relationship between alcohol use and PEth.
- More cost-effective analytical procedures are needed to fully realize the clinical potential of newer alcohol consumption biomarkers such as PEth.

References

Alling C, Gustavsson L, Anggard E. An abnormal phospholipid in rat organs after ethanol treatment. FEBS Lett. 1983;152:24–8.

Aradottir S, Asanovska G, Gjerss S, et al. Phosphatidylethanol (PEth) concentrations in blood are correlated to reported alcohol intake in alcohol-dependent patients. Alcohol Alcohol. 2006;41:431–7.

Arndt T, van der Meijden BB, Wielders JP. Atypical serum transferrin isoform distribution in liver cirrhosis studied by HPLC, capillary electrophoresis and transferrin genotyping. Clin Chim Acta. 2008;394:42–6.

Babor TF, de la Fuente JR, Saunders J, Grant M. AUDIT. The alcohol use disorders identification test. Guidelines for use in primary health care. Geneva: World Health Organization; 1992.

Barve A, Khan R, Marsano L, et al. Treatment of alcoholic liver disease. Ann Hepatol. 2008;7:5–15.

Bergstrom JP, Helander A. Clinical characteristics of carbohydrate-deficient transferrin (% disialotransferrin) measured by HPLC: sensitivity, specificity, gender effects, and relationship with other alcohol biomarkers. Alcohol Alcohol. 2008;43:436–41.

Bradley KA, Debenedetti AF, Volk RJ, et al. AUDIT-C as a brief screen for alcohol misuse in primary care. Alcohol Clin Exp Res. 2007;31:1208–17.
Cohen JI, Nagy LE. Pathogenesis of alcoholic liver disease: interactions between parenchymal and non-parenchymal cells. J Dig Dis. 2011;12:3–9.
Conigrave KM, Davies P, Haber P, Whitfield JB. Traditional markers of excessive alcohol use. Addiction. 2003;98 Suppl 2:31–43.
Society of Hair Testing, Consensus for the use of alcohol markers in hair for assessment of both abstinence and chronic excessive alcohol consumption. Bordeaux. 2014.
Del Boca FK, Darkes J. The validity of self-reports of alcohol consumption: state of the science and challenges for research. Addiction. 2003;98 Suppl 2:1–12.
Dimartini A, Day N, Lane T, et al. Carbohydrate deficient transferrin in abstaining patients with end-stage liver disease. Alcohol Clin Exp Res. 2001;25:1729–33.
Dresen S, Weinmann W, Wurst FM. Forensic confirmatory analysis of ethyl sulfate – a new marker for alcohol consumption – by liquid-chromatography/electrospray ionization/tandem mass spectrometry. J Am Soc Mass Spectrom. 2004;15:1644–8.
Erim Y, Bottcher M, Dahmen U, et al. Urinary ethyl glucuronide testing detects alcohol consumption in alcoholic liver disease patients awaiting liver transplantation. Liver Transpl. 2007;13:757–61.
Gnann H, Engelmann C, Skopp G, et al. Identification of 48 homologues of phosphatidylethanol in blood by LC-ESI-MS/MS. Anal Bioanal Chem. 2010;396:2415–23.
Gnann H, Weinmann W, Thierauf A. Formation of phosphatidylethanol and its subsequent elimination during an extensive drinking experiment over 5 days. Alcohol Clin Exp Res. 2012;36:1507–11.
Gunnarsson T, Karlsson A, Hansson P, et al. Determination of phosphatidylethanol in blood from alcoholic males using high-performance liquid chromatography and evaporative light scattering or electrospray mass spectrometric detection. J Chromatogr B Biomed Sci Appl. 1998;705:243–9.
Gustavsson L. Phosphatidylethanol formation: specific effects of ethanol mediated via phospholipase D. Alcohol Alcohol. 1995;30:391–406.
Hahn JA, Dobkin LM, Mayanja B, et al. Phosphatidylethanol (PEth) as a biomarker of alcohol consumption in HIV-positive patients in sub-Saharan Africa. Alcohol Clin Exp Res. 2012;36:854–62.
Hansson P, Caron M, Johnson G, et al. Blood phosphatidylethanol as a marker of alcohol abuse: levels in alcoholic males during withdrawal. Alcohol Clin Exp Res. 1997;21:108–10.
Hartmann S, Aradottir S, Graf M, et al. Phosphatidylethanol as a sensitive and specific biomarker: comparison with gamma-glutamyl transpeptidase, mean corpuscular volume and carbohydrate-deficient transferrin. Addict Biol. 2007;12:81–4.
Holbrook PG, Pannell LK, Murata Y, Daly JW. Molecular species analysis of a product of phospholipase D activation. Phosphatidylethanol is formed from phosphatidylcholine in phorbol ester- and bradykinin-stimulated PC12 cells. J Biol Chem. 1992;267:16834–40.
Jain J, Evans JL, Briceno A, et al. Comparison of phosphatidylethanol results to self-reported alcohol consumption among young injection drug users. Alcohol Alcohol. 2014;49:520–4.
Kim WR, Lake JR, Smith JM, et al. OPTN/SRTR 2013 annual data report: liver. Am J Transplant. 2015;15 Suppl 2:1–28.
Kwak HS, Han JY, Ahn HK, et al. Blood levels of phosphatidylethanol in pregnant women reporting positive alcohol ingestion, measured by an improved LC-MS/MS analytical method. Clin Toxicol. 2012;50:886–91.
Morini L, Politi L, Groppi A, et al. Determination of ethyl glucuronide in hair samples by liquid chromatography/electrospray tandem mass spectrometry. J Mass Spectrom. 2006;41:34–42.
Mueller GC, Fleming MF, Lemahieu MA, et al. Synthesis of phosphatidylethanol – a potential marker for adult males at risk for alcoholism. Proc Natl Acad Sci U S A. 1988;85:9778–82.
Nissinen AE, Makela SM, Vuoristo JT, et al. Immunological detection of in vitro formed phosphatidylethanol – an alcohol biomarker – with monoclonal antibodies. Alcohol Clin Exp Res. 2008;32:921–8.

Pragst F, Rothe M, Moench B, et al. Combined use of fatty acid ethyl esters and ethyl glucuronide in hair for diagnosis of alcohol abuse: interpretation and advantages. Forensic Sci Int. 2010;196:101–10.

Sobell L, Sobell M. Timeline follow-back: a technique for assessing self-reported ethanol consumption. In: Litten RZ, Allen JP, editors. Measuring alcohol consumption: psychosocial and biological methods. Totowa: Humana Press; 1992. p. 41–72.

Staufer K, Andresen H, Vettorazzi E, et al. Urinary ethyl glucuronide as a novel screening tool in patients pre- and post-liver transplantation improves detection of alcohol consumption. Hepatology. 2011;54:1640–9.

Stewart SH, Reuben A, Brzezinski WA, et al. Preliminary evaluation of phosphatidylethanol and alcohol consumption in patients with liver disease and hypertension. Alcohol Alcohol. 2009;44:464–7.

Stewart SH, Law TL, Randall PK, Newman R. Phosphatidylethanol and alcohol consumption in reproductive age women. Alcohol Clin Exp Res. 2010;34:488–92.

Stewart SH, Koch DG, Burgess DM, et al. Sensitivity and specificity of urinary ethyl glucuronide and ethyl sulfate in liver disease patients. Alcohol Clin Exp Res. 2013a;37:150–5.

Stewart SH, Koch DG, Willner IR, et al. Hair ethyl glucuronide is highly sensitive and specific for detecting moderate-to-heavy drinking in patients with liver disease. Alcohol Alcohol. 2013b;48:83–7.

Stewart SH, Koch DG, Willner IR, et al. Validation of blood phosphatidylethanol as an alcohol consumption biomarker in patients with chronic liver disease. Alcohol Clin Exp Res. 2014;38:1706–11.

Szabo G, Wands JR, Eken A, et al. Alcohol and hepatitis C virus – interactions in immune dysfunctions and liver damage. Alcohol Clin Exp Res. 2010;34:1675–86.

UKPDS Writing Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet. 1998;352:837–53.

Varga A, Alling C. Formation of phosphatidylethanol in vitro in red blood cells from healthy volunteers and chronic alcoholics. J Lab Clin Med. 2002;140:79–83.

Varga A, Hansson P, Johnson G, Alling C. Normalization rate and cellular localization of phosphatidylethanol in whole blood from chronic alcoholics. Clin Chim Acta. 2000;299:141–50.

Wrighton SA, Pai JK, Mueller GC. Demonstration of two unique metabolites of arachidonic acid from phorbol ester-stimulated bovine lymphocytes. Carcinogenesis. 1983;4:1247–51.

Wurst FM, Seidl S, Ladewig D, et al. Ethyl glucuronide: on the time course of excretion in urine during detoxification. Addict Biol. 2002;7:427–34.

Wurst FM, Alexson S, Wolfersdorf M, et al. Concentration of fatty acid ethyl esters in hair of alcoholics: comparison to other biological state markers and self reported-ethanol intake. Alcohol Alcohol. 2004a;39:33–8.

Wurst FM, Wiesbeck GA, Metzger JW, Weinmann W. On sensitivity, specificity, and the influence of various parameters on ethyl glucuronide levels in urine – results from the WHO/ISBRA study. Alcohol Clin Exp Res. 2004b;28:1220–8.

Wurst FM, Thon N, Aradottir S, et al. Phosphatidylethanol: normalization during detoxification, gender aspects and correlation with other biomarkers and self-reports. Addict Biol. 2010;15:88–95.

Yoon Y, Yi H. Surveillance report # 88- liver cirrhosis mortality in the United States, 1970–2007. US Department of Health and Human Services, Public Health Service, National Institutes of Health; 2010.

Zakhari S, Li TK. Determinants of alcohol use and abuse: impact of quantity and frequency patterns on liver disease. Hepatology. 2007;46:2032–9.

Circulating Extracellular Vesicles as Liver Biomarkers

26

Qiang Shi

Contents

Abstract

Extracellular vesicles (EV) released by various types of cells are detectable in body fluids such as blood and urine. Both clinical and animal studies showed that circulating EVs were elevated in response to many types of liver diseases, such as hepatocellular carcinoma (HCC), nonalcoholic fatty liver disease, acute liver failure, liver cirrhosis, hepatic ischemia-reperfusion (I/R) injury, and drug-induced liver injury. The increase in subgroups of EVs and certain EV molecules was found to be of diagnostic and prognostic value for some liver diseases such as HCC and liver cirrhosis. Some microRNA species were solely increased in serum EVs but not the whole serum under disease states, indicating that circulating EVs

Disclaimer: The information in these materials is not a formal dissemination of information by FDA and does not represent agency position or policy.

Q. Shi (✉)
Division of Systems Biology, National Center for Toxicological Research, The United States Food and Drug Administration, Jefferson, AR, USA
e-mail: qiang.shi@fda.hhs.gov

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_38

may provide more sensitive liver biomarkers. Blood EVs from patients appeared to play a detrimental role contributing disease progression. Blocking blood EV elevation by pharmacological approaches afforded protection against liver damage. Circulating EVs are emerging as a rich source of new biomarkers for liver diseases.

Keywords

Extracellular vesicles • Biomarkers • Hepatocellular carcinoma • Nonalcoholic fatty liver disease • Acute liver failure • Liver cirrhosis • Hepatic ischemia-reperfusion (I/R) injury • Drug-induced liver injury

List of Abbreviations

ALF	Acute liver failure
ALT	Alanine aminotransferase
AFP	Alpha-fetoprotein
ASGPR1	Asialoglycoprotein receptor
DILI	Drug-induced liver injury
EV	Extracellular vesicles
HepB	Hepatitis B
HepC	Hepatitis C
HCC	Hepatocellular carcinoma
I/R	Ischemia-reperfusion
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
ROCK1	Rho-associated, coiled-coil-containing protein kinase 1

Key Facts of Extracellular Vesicles

- Extracellular vesicles (EVs) were first described in 1946 as "dust" produced by platelets in normal plasma. EVs were considered as cellular waste for many years.
- The 2013 Nobel Prize in Physiology or Medicine was awarded to three scientists who study vesicles including EVs.
- A professional organization on EVs studies, the International Society for Extracellular Vesicles (ISEV), was established in 2011. ISEV launched its official journal, *Journal of Extracellular Vesicles*, in 2012.
- EVs are produced by almost all cells and circulate in the body. EVs can be isolated and detected in all types of body fluids.
- EVs are of different sizes and forms. EVs contain RNAs, proteins, and lipids.
- When liver diseases occur, both EVs produced by liver cells and non-liver cells are changed. These changes can be detected in the blood and urine, and the pattern of changes may help determine the severity of liver disorders.
- The changes in blood EVs can be prevented by pharmacological approaches, and this intervention is beneficial to liver diseases.

Definitions of Words and Terms

Acetaminophen overdose	Acetaminophen is active ingredient of many pain killers such as Tylenol. Though acetaminophen is a very safe drug, it can cause serious liver injury when taken too much. Acetaminophen overdose is a main reason for acute liver failure.
Acute liver failure (ALF)	ALF refers to the very rapid loss of liver functions that causes mental problems and bleeding disorders. ALF is life threatening and often requires liver transplantation.
Biomarkers	A biomarker usually refers to a molecule that can be quantified to reflect the occurrence and/or progress of specific diseases. An ideal biomarker is expected to be both sensitive and specific.
Extracellular vesicles (EVs)	EVs are small particles released by all types of cells. The size of EVs ranges from 50 to 5,000 nm in diameter. EVs are composed of proteins, lipids, and nucleic acids. EVs circulate in the blood playing an important role in cell-to-cell communications.
Hepatic ischemia-reperfusion (I/R) injury	Hepatic I/R injury usually occurs during liver surgery. When the blood flow through the liver is reduced, the liver will be damaged due to lack of oxygen and other nutrients. However, such damage will be made worse when the blood flow was restored to normal.
Hepatocellular carcinoma (HCC)	HCC is a type of cancer that originally occurs in the liver. HCC is actually the most common form of liver cancer. Hepatitis B or C patients are more likely to develop HCC.
Hepatocytes	Hepatocytes are the main type of cells in the liver. Hepatocytes perform the main functions of the liver.
Liver cirrhosis	Liver cirrhosis refers to the serious scarring of liver tissues. Liver scars do not work as normal liver cells and therefore liver functions are compromised in the long run. Those drinking too much alcohol and those with hepatitis B or C are more likely to have liver cirrhosis.
MicroRNAs	MicroRNAs are a type of short RNAs with about 20 nucleotides. MicroRNAs regulate the expression of many genes. MicroRNAs are very stable and therefore are considered as good disease biomarkers.

Nonalcoholic fatty liver disease (NAFLD)	NAFLD refers to the buildup of fat in liver cells in those who do not drink alcohol. NAFLD is a common liver disorder, and the main cause of NAFLD is obesity. The liver cells do not have inflammation or damage in NAFLD patients.
Nonalcoholic steatohepatitis (NASH)	NASH is a liver disorder caused by the accumulation of too much fat that leads to inflammation and damage in the liver. NASH occurs in people who do not drink alcohol.

Introduction

Extracellular vesicles (EVs), first discovered in 1946, generally refer to membrane-enclosed vesicles secreted by essentially all types of cells including prokaryotic cells. The secretion of EVs appears to be highly conserved in evolution (Yanez-Mo et al. 2015). Both healthy and abnormal cells produce EVs in a constant manner, though the number, morphology, and constituents of EV are changing with the environmental conditions. EVs were found to be cup shaped or round shaped or of irregular morphology. As with other biological entities, EVs are composed of proteins, nucleic acids, and lipids. At least 41,860 proteins, 7,540 RNAs, and 1,116 lipid molecules have been confidently identified in EVs (Keerthikumar et al. 2016). However, the function of EV molecules remains largely unknown. As of April 26, 2016, the PubMed contained 9,212 abstracts associated with EVs. However, only 295 abstracts were related to both EVs and the "liver." This indicates that the study of liver EVs is still at the very early stage. It should be pointed out that EVs is the preferred term for studies involving different types of secreted vesicles including exosomes, microvesicles/microparticles/ectosomes, and apoptotic bodies (Gould and Raposo 2013). The main characteristics of these subgroups of EVs are summarized in Table 1. All these subgroups of EVs in the circulation have been studied in liver diseases for the discovery of new biomarkers.

EVs secreted by hepatocytes have been extensively characterized using systems biology approaches such as proteomics and genomics. The majority of genes and proteins identified in hepatocyte EVs were similar to those in EVs from other cell types, though some molecules appeared to be liver EV specific (Royo et al. 2013; Rodriguez-Suarez et al. 2014; Conde-Vancells et al. 2008). Figure 1 shows two typical protein markers for EVs isolated from primary cultured hepatocytes. This raised the possibility that liver EVs released into the circulation may serve as a potential source for highly specific biomarkers of liver diseases. Lack of specificity, and to a lesser extent sensitivity, is a key drawback of many standard liver biomarkers such as those for drug-induced liver injury (DILI) (Shi et al. 2010). Indeed, circulating EVs have been shown to be not only a potential source for more specific liver biomarkers but also an appealing target for therapeutic interventions.

Table 1 Subgroups of EVs. The main characteristics of three types of commonly studied EVs are summarized in the table

Subgroups of EVs	Biogenesis	Size (nm)	Morphology	Ref
Exosomes	Endosomal network fused with plasma membrane	50–100	Cup shaped	(Yanez-Mo et al. 2015; van der Pol et al. 2012; Andaloussi et al. 2013)
Microvesicles/ microparticles/ ectosomes	Budding and fission of plasma membrane	20–1,000	Cup shaped	
Apoptotic bodies	Blebs of apoptotic cells	1,000–5,000	Irregular	

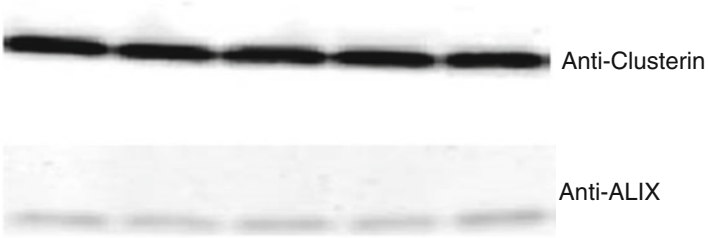

Fig. 1 Protein markers for EVs isolated from primary cultured rat hepatocytes. EVs were isolated from primary hepatocytes prepared from five different rats. Western blot was used to detect the EV protein markers clusterin and ALIX (unpublished data)

When liver disease occurred, not only the morphology but also the components of hepatocyte EVs were changed. These altered hepatocyte EVs together with EVs of non-hepatocyte origin were released into the circulation and can be used as new liver biomarkers. Though EVs are detectable in various types of body fluids, only these in the blood and urine have been explored as liver biomarkers. Findings regarding circulating EVs as DILI biomarkers have been summarized in a recent review (Yang et al. 2014) and therefore will not be covered in this chapter.

Hepatocellular Carcinoma (HCC)

HCC is the most common form of liver cancer, and the most studied area on circulating EVs as liver biomarkers is HCC. This is not unexpected in that EVs from cancer cells have distinct constituents and circulating tumor EVs have been extensively characterized for discovering new cancer biomarkers. Though circulating EVs as cancer biomarkers were recognized as early as in the 1970s (Taylor and Shah 2015), the first study on blood EVs in HCC patients was not published until

2008 (Brodsky et al. 2008). It was found that the number of total blood EVs was increased by fivefold in HCC/HepC (HepC, hepatitis C infection) patients as compared with control subjects, and such elevation was relatively small, that is, about threefold, in HepC only patients. Though the total number of blood EVs did not directly reflect tumor size, the level of blood EVs from hepatocytes and endothelial cells, but not these from apoptotic cells, correlated well with tumor size (Brodsky et al. 2008). Interestingly, circulating EVs were first increased and then decreased after surgery. It appeared that the number of circulating EVs was associated with HCC clinical outcome, as a patient who later died had persistent increases of EVs prior to and after liver transplantation, and patients whose blood EV level returned to normal showed little clinical complications after liver transplantation. However, the small sample size ($n = 8$) made it difficult to draw a statistically meaningful conclusion (Brodsky et al. 2008). A more recent study confirmed and expanded some of these early findings. Using blood samples from 55 HCC patients, it was demonstrated that serum EV levels were significant increased and the changes correlated well with tumor size prior to treatment, and surgery treatment remarkably reduced EV numbers in the blood (Wang et al. 2013). Notably, serum EV levels showed diagnostic value in differentiating HCC stages (Wang et al. 2013).

As the number and concentration of blood EVs cannot be accurately quantified without extensive experience, measuring specific molecules in EVs, particularly the stable ones such as microRNAs, would likely provide more reliable and reproducible results. A recent study examined microRNA-21 levels in serum EVs from HCC patients (Wang et al. 2014). The rationale for selecting microRNA-21 was that it is a highly expressed microRNA species in numerous cancer cells. It was found that microRNA-21 in serum EVs was increased by over 16-fold in HCC patients as compared with healthy subjects. In contrast, serum EV microRNA-21 was increased by about fourfold in patients with hepatitis B infection. However, the microRNA-21 in whole serum was nearly unchanged, indicating that microRNA-21 was enriched in EVs (Wang et al. 2014). This observation demonstrates the unique advantage of using blood EVs as compared to whole blood. The additional value of microRNA-21 in EVs was that its level showed good correlation to HCC stages and the development of cirrhosis (Wang et al. 2014).

To identify more candidate biomarkers for HCC, serum EV microRNAs were detected in a more comprehensive manner. Specially, ten microRNAs that were dysregulated in HCC tissues were measured in serum EVs from HCC patients. As compared to patients with chronic hepatitis B, four EV microRNAs including miR-18a, miR-221, miR-222, and miR-22 were increased in HCC patients, and four EV microRNAs including miR-101, miR-106b, miR-122, and miR-195 were decreased, and the remaining two microRNAs miR-21 and miR-93 showed no difference (Sohn et al. 2015). In line with a previous report, the tested serum microRNAs showed no difference among disease groups, indicating that microRNAs enriched in EV are more sensitive in detecting HCC. A significant drawback of this study is that there were no samples from healthy subjects.

Recently, blood EV microRNAs from HCC patients were comprehensively analyzed using microRNA arrays. It was found that one microRNA, miR-1246,

was remarkably increased and one microRNA, hsa-miR-718, was significantly decreased in serum EVs from HCC patients with recurrence as compared with those having no recurrence (Sugimachi et al. 2015). The level of hsa-miR-718, but not miR-1246, showed good correlation to tumor size, histological differentiation, and recurrence-free survival rate (Sugimachi et al. 2015).

Perturbation of circulating EVs was also observed in animal models for HCC. In rats treated with diethylnitrosamine, a well-established HCC model, two serum EV microRNAs, miRNA-10b and miRNA-21, were increased at later HCC stages. Combining the expressional levels of serum EV microRNAs and circulating microRNAs showed strong predictive value in the development of HCC in rats (Liu et al. 2015).

In clinical studies mentioned above, HCC was often associated with chronic hepatitis B or C infection. In most cases, an elevation of blood EVs was also observed in chronic hepatitis B or C patients, but the extent of changes was different.

A significant challenge in using circulating EV microRNAs as HCC biomarkers is that no standard normalization method is currently available. A recent study showed that when serum EV miR-21 level was normalized to a group of microRNAs including miR-221, miR-191, miR-181a, and miR-26a, its expression was higher in chronic hepatitis B patients than in HCC patients. However, such difference disappeared when miR-181c or *U6 (CCG-1)* was used as normalizers (Li et al. 2015). Regardless of the method for data normalization, serum EV miR-21 levels were significantly higher in HCC patients than in healthy subjects, though the difference between HCC patients and hepatitis B patients was no longer statistically different when some normalizers were used (Li et al. 2015).

In the abovementioned studies, blood EVs and EV microRNAs consistently outperformed traditional biomarkers such as alpha-fetoprotein (AFP) in HCC detection. Circulating EVs have been demonstrated to be a rich source for novel biomarkers of HCC. To date, only microRNAs in blood EV have been studied in HCC patients. Other molecules, such as proteins and long noncoding RNAs that have been studied in the whole blood for HCC detection, await further characterization regarding their expression profiles in circulating EVs (Table 2).

Nonalcoholic Fatty Liver Disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH)

NAFLD is the most common form of chronic liver diseases. NASH is the more severe form of NAFLD. Circulating EVs as biomarkers for NAFLD and NASH have studied using both animal models and clinical samples (Table 3).

In mouse models of NAFLD, blood EVs were found to be significantly increased, and the alterations appeared to be time dependent and correlated well with the histopathological features of disease severity (Povero et al. 2014). Further characterization of the increased blood EVs showed that the liver-enriched microRNAs miR-122 and miR-192 were remarkably increased, which was accompanied by a notable decrease of these two microRNAs in the liver tissue, indicating that at least part of blood EVs were produced by the liver. This is further confirmed by the

Table 2 Circulating EVs as HCC biomarkers. Summary of published data on using circulating EVs as HCC biomarkers

Study design	Approaches for EV characterization	Major findings	Ref
8 HCC/HepC and 8 HepC patients[a]; 5 control subjects without HCC or HepC	Antibody labeling followed by flow cytometry	EVs were increased in both HCC/HepC and HepC patients; EVs from hepatic and endothelial cells correlated well with HCC tumor size	(Brodsky et al. 2008)
55 HCC and 40 liver cirrhosis patients; 21 healthy subjects	Ultracentrifugation and protein assay (bicinchoninic acid method)	EVs were increased in HCC patients, and EV levels were predictive of HCC stages as determined by receiver-operating characteristic analysis	(Wang et al. 2013)
30 HCC and 30 HepB patients; 30 healthy subjects	Exosome isolation kit followed by electron microscopy and microRNA detection	MicroRNA-21 in serum EVs was increased in HCC patients, and its level correlated well with tumor stage and cirrhosis	(Wang et al. 2014)
20 HCC, 20 HepB patients, and 20 cirrhosis patients; no healthy subjects	Exosome isolation kit followed by microRNA detection	Four microRNAs were increased and four were decreased in HCC patients as compared to HepB patients	(Sohn et al. 2015)
65 HCC patients with liver transplantation	Ultracentrifugation followed by electron microscopy and microRNA detection	MicroRNA-718 in serum EVs predicted HCC recurrence after liver transplantation	(Sugimachi et al. 2015)
50 HCC and 50 HepB patients; 50 healthy subjects	Exosome isolation kit followed by electron microscopy and microRNA detection	MicroRNA-21 in serum EVs was increased in HCC patients; data normalization methods affected the result	(Li et al. 2015)
108 rats treated with diethylnitrosamine	Ultracentrifugation followed by electron microscopy and microRNA detection	MicroRNAs in blood EVs were more sensitive in detecting chemical-induced liver cancer in rats	(Liu et al. 2015)

[a]All patients had cirrhosis due to hepatitis C (HepC) infection and received liver transplantation. HepB, hepatitis B infection

significant increase of hepatocyte-specific protein asialoglycoprotein receptor (ASGPR1) in the blood EVs from NAFLD mice. Extensive analysis of blood EVs by proteomics approach showed that 25 EV proteins were increased in NAFLD mice and can be used to help the diagnosis of NAFLD (Povero et al. 2014).

In a mouse model of NASH, the concentration of serum EVs was increased by twofold, and some EV-associated proteins, such as the hepatocyte-specific enzyme CYP2E1, were also upregulated in the circulating EVs (Hirsova et al. 2016). The overproduced circulating EVs appeared to be functional, as EVs from both NASH

Table 3 Circulating EVs as NAFLD/NASH biomarkers. Summary of published data on using circulating EVs as NAFLD/NASH biomarkers. HepC, hepatitis C infection

Study design	Approaches for EV characterization	Major findings	Ref
67 patients with NAFLD or NASH; 42 patients with HepC; 44 healthy subjects	Ultracentrifugation followed by antibody labeling and flow cytometry analysis	Blood EVs predicted NAFLD or NASH by receiver-operating characteristic analysis; subgroups of blood EVs correlated well with disease severity	(Kornek et al. 2012)
Mouse models of NAFLD produced by choline deficient L-amino acid and high-fat diets	Ultracentrifugation followed by electron microscopy, antibody labeling, microRNA detection, and proteomics	Protein profiles in blood EVs predicted the development of NAFLD; Blood EVs levels reflected the severity of NAFLD; blood EVs contained microRNAs produced by hepatocytes	(Povero et al. 2014)
Mouse model of NASH produced by high-fat diets	Ultracentrifugation followed by electron microscopy and nanoparticle tracking analysis	Serum EVs were increased in NASH mouse, and this was prevented by a pharmacological approach which also afforded protection against liver injury	(Hirsova et al. 2016)

patients and NASH mice activated macrophages causing enhanced cytokine production (Hirsova et al. 2016). Interestingly, when EV overproduction in the circulation was prevented by fasudil, a chemical inhibitor of rho-associated, coiled-coil-containing protein kinase 1 (ROCK1), the macrophage-mediated liver inflammation was reduced (Hirsova et al. 2016). Though increased circulating EVs seemed detrimental to liver functions, the responsible molecules and pathways have not been identified. Nevertheless, circulating EVs do have values in not only helping the diagnosis of NASH but also monitoring the therapeutic responses of anti-NASH medications.

In a clinical study with 67 NAFLD or NASH patients and 44 healthy subjects, it was found that blood EVs were significantly increased and blood EVs levels correlated well with the gold standard criteria for the severity of inflammation and apoptosis in NAFLD and NASH. This study also noted a slight difference between EVs from plasma and serum (Kornek et al. 2012).

Acute Liver Failure (ALF) and Acute Liver Injury

ALF is a relatively rare liver condition with a high mortality, and liver transplantation is often needed for treatment. In a recent prospective study involving 50 patients with ALF, plasma EVs were found to be increased by nearly 20-fold in ALF patients

as compared with healthy subjects. The plasma level of a subgroup of EVs with the size of 280–640 nm was found to be predictive of patient outcome (alive or dead) (Stravitz et al. 2013). In this study, the etiology of ALF-included acetaminophen overdose, hepatitis B infection, autoimmune hepatitis, and mushroom poisoning (Stravitz et al. 2013) indicates that circulating EVs can be used to detect ALF caused by various reasons. In smaller study involving 10 patients who developed acute liver injury, plasma EVs were also found to be increased, though the fold change was relatively small, that is, two to three folds (Schmelzle et al. 2013).

Liver Cirrhosis

Liver cirrhosis is a severe condition of scarring of the liver and loss of liver function occurring at the terminal stages of chronic liver disease. In a relatively large clinical study with 91 liver cirrhosis patients and 30 healthy subjects, it was found that blood EVs were significantly elevated regardless of the cause of liver cirrhosis, and a subpopulation of these EVs was shown to be from hepatocytes. The elevation of hepatocyte-derived EVs in the blood was associated with the severity of cirrhosis and systemic inflammation, indicating the circulating EVs may serve as new biomarkers for the grading of liver cirrhosis. Interestingly, women appeared to have a slightly higher level of certain blood EVs as compared to men (Rautou et al. 2012). The blood EVs from liver cirrhosis patients may contribute to the pathogenesis of liver cirrhosis, as these EVs caused vascular hyporeactivity and decreased arterial blood pressure which may contribute to arterial vasodilation associated with portal hypertension (Rautou et al. 2012). Targeting these detrimental EVs may provide therapeutic benefits, but this has not been examined in details with liver cirrhosis patients.

Hepatic Ischemia-Reperfusion (I/R) Injury

Hepatic I/R injury refers to the heightened hypoxic cellular damage following the restoration of liver blood flow during surgical procedures. Changes in circulating EVs for I/R injury detection have only been explored in animal models. In a mouse model of hepatic I/R injury, plasma EVs were found to be significantly increased. Subpopulation analysis showed that EVs from platelets and neutrophils were elevated at the acute injury stage while those from endothelial were increased at a later stage, indicating the former may serve as injury biomarkers and the latter may better reflect liver regeneration (Freeman et al. 2014).

Another mouse study found that blood EVs began to increase 15–30 min after postischemic reperfusion when serum alanine aminotransferase (ALT) was unchanged, and the elevation persisted and reached the peak level at 24 h (Teoh et al. 2014). The origin of elevated blood EVs was dependent on time course of liver injury. At 30 min after reperfusion, the blood EVs were mainly from endothelial cells, platelets, and neutrophils, while at 2 h and later, the blood EVs appeared to be

mainly from Kupffer cells/macrophages (Teoh et al. 2014). These circulating EVs after I/R injury were able to trigger inflammatory response causing further liver injury. Interestingly, blocking the overproduction of circulating EVs by intravenous injection of diannexin, a synthetic human recombinant homodimer of annexin V, afforded significant protection against hepatic I/R injury in mice (Teoh et al. 2014). The clinical value of using circulating EVs for detecting hepatic I/R injury awaits further investigation.

Potential Applications to Prognosis, Other Diseases, or Conditions

The magnitude of changes in circulating EVs under different liver disease conditions was relatively small as compared to serum ALT. Therefore they may not be highly sensitive in detecting liver diseases. However, blood EVs appeared to be more specific and more informative than traditional liver biomarkers. Evidence is emerging that blood EVs of certain origins and their selective constituents such as microRNAs have the potential to predict HCC stages and recurrence (Brodsky et al. 2008; Liu et al. 2015; Sohn et al. 2015; Sugimachi et al. 2015; Wang et al. 2013, 2014), and plasma EVs of certain sizes may help predict if the ALF patients will die or survive (Stravitz et al. 2013). As for NAFLD and NASH, the blood EVs were predictive of disease severity (Kornek et al. 2012). However, the lack of standardized methods for EV isolation and data normalization makes it difficult to compare and evaluate the result from different groups. The methods for isolating circulating EVs are evolving with time. The reproducibility of published data needs to be confirmed using more patient samples, as a common drawback of existing data is that only a limited number of patients, that is, usually less than 100, were examined. The likely reason is that EV isolation from blood requires expensive instruments such as an ultracentrifuge and the whole process is rather time-consuming. The characterization of isolated blood EVs is also a challenging task. Improvement in EV isolation method is urgently needed for the wide application of circulating EV-based liver biomarkers.

Accumulating data suggest that circulating EVs play a functional role in mediating the pathogenesis of various liver diseases (Lemoinne et al. 2014). Therapeutic interventions very likely will affect the blood EV profiles. Interestingly, intrasplenic administration of EVs from certain cell types afforded protection against acute liver injury induced by chemicals, possible via the activation of regenerative process (Tan et al. 2014). This should be taken into consideration when circulating EVs are to be used for prognosis of liver diseases.

In addition to liver disorders, blood EVs have been shown to be disrupted under numerous disease conditions, particularly various types of cancers. A comprehensive study comparing the alterations of circulating EVs under different disease states is not available, making it difficult to assess the specificity of blood EVs as liver disease biomarkers. Nevertheless, organ-specific molecules in circulating EVs may hold the promise of serving as disease-specific biomarkers.

Summary Points

- This chapter focuses on circulating extracellular vesicles (EVs) as biomarkers for various liver diseases.
- EVs are produced by various cell types and are released in the blood and urine.
- The number, morphology, and constituents of EVs are altered when liver diseases occur.
- Circulating EVs are elevated in patients or animals with hepatocellular carcinoma (HCC), nonalcoholic fatty liver disease (NAFLD), acute liver failure (ALF) and acute liver injury, liver cirrhosis, hepatic ischemia-reperfusion (I/R) injury, and drug-induced liver injury (DILI).
- Blood EVs originated from hepatocytes have the potential to predict liver disease stages and outcome (alive or dead).
- Certain proteins and microRNAs in blood EVs may serve as new biomarkers for liver diseases.
- Methods for the isolation of circulating EVs need to be improved and standardized.

References

Andaloussi ELA, Mager I, Breakefield XO, et al. Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov. 2013;12:347–57.

Brodsky SV, Facciuto ME, Heydt D, et al. Dynamics of circulating microparticles in liver transplant patients. J Gastrointestin Liver Dis. 2008;17:261–88.

Conde-Vancells J, Rodriguez-Suarez E, Embade N, et al. Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. J Proteome Res. 2008;7:5157–66.

Freeman CM, Quillin 3rd RC, Wilson GC, et al. Characterization of microparticles after hepatic ischemia-reperfusion injury. PLoS One. 2014;9:e97945.

Gould SJ, Raposo G. As we wait: coping with an imperfect nomenclature for extracellular vesicles. J Extracell Vesicles. 2013;2:201389.

Hirsova P, Ibrahim SH, Krishnan A, et al. Lipid-induced signaling causes release of inflammatory extracellular vesicles from hepatocytes. Gastroenterolog. 2016;150:956–67.

Keerthikumar S, Chisanga D, Ariyaratne D, et al. ExoCarta: a web-based compendium of exosomal cargo. J Mol Biol. 2016;428:688–92.

Kornek M, Lynch M, Mehta SH, et al. Circulating microparticles as disease-specific biomarkers of severity of inflammation in patients with hepatitis C or nonalcoholic steatohepatitis. Gastroenterology. 2012;143:448–58.

Lemoinne S, Thabut D, Housset C, et al. The emerging roles of microvesicles in liver diseases. Nat Rev Gastroenterol Hepatol. 2014;11:350–61.

Li Y, Zhang L, Liu F, et al. Identification of endogenous controls for analyzing serum exosomal miRNA in patients with hepatitis B or hepatocellular carcinoma. Dis Markers. 2015;2015:893594.

Liu WH, Ren LN, Wang X, et al. Combination of exosomes and circulating microRNAs may serve as a promising tumor marker complementary to alpha-fetoprotein for early-stage hepatocellular carcinoma diagnosis in rats. J Cancer Res Clin Oncol. 2015;141:1767–78.

Povero D, Eguchi A, Li H, et al. Circulating extracellular vesicles with specific proteome and liver microRNAs are potential biomarkers for liver injury in experimental fatty liver disease. PLoS One. 2014;9:e113651.

Rautou PE, Bresson J, Sainte-Marie Y, et al. Abnormal plasma microparticles impair vasoconstrictor responses in patients with cirrhosis. Gastroenterology. 2012;143:166–76.
Rodriguez-Suarez E, Gonzalez E, Hughes C, et al. Quantitative proteomic analysis of hepatocyte-secreted extracellular vesicles reveals candidate markers for liver toxicity. J Proteomics. 2014;103:227–40.
Royo F, Schlangen K, Palomo L, et al. Transcriptome of extracellular vesicles released by hepatocytes. PLoS One. 2013;8:e68693.
Schmelzle M, Splith K, Andersen LW, et al. Increased plasma levels of microparticles expressing CD39 and CD133 in acute liver injury. Transplantation. 2013;95:63–9.
Shi Q, Hong H, Senior J, et al. Biomarkers for drug-induced liver injury. Expert Rev Gastroenterol Hepatol. 2010;4:225–34.
Sohn W, Kim J, Kang SH, et al. Serum exosomal microRNAs as novel biomarkers for hepatocellular carcinoma. Exp Mol Med. 2015;47:e184.
Stravitz RT, Bowling R, Bradford RL, et al. Role of procoagulant microparticles in mediating complications and outcome of acute liver injury/acute liver failure. Hepatology. 2013;58:304–13.
Sugimachi K, Matsumura T, Hirata H, et al. Identification of a bona fide microRNA biomarker in serum exosomes that predicts hepatocellular carcinoma recurrence after liver transplantation. Br J Cancer. 2015;112:532–8.
Tan CY, Lai RC, Wong W, et al. Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. Stem Cell Res Ther. 2014;5:76.
Taylor DD, Shah S. Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes. Methods. 2015;87:3–10.
Teoh NC, Ajamieh H, Wong HJ, et al. Microparticles mediate hepatic ischemia-reperfusion injury and are the targets of Diannexin (ASP8597). PLoS One. 2014;9:e104376.
van der Pol E, Boing AN, Harrison P, et al. Classification, functions, and clinical relevance of extracellular vesicles. Pharmacol Rev. 2012;64:676–705.
Wang W, Li H, Zhou Y, et al. Peripheral blood microvesicles are potential biomarkers for hepatocellular carcinoma. Cancer Biomark. 2013;13:351–7.
Wang H, Hou L, Li A, et al. Expression of serum exosomal microRNA-21 in human hepatocellular carcinoma. Biomed Res Int. 2014;2014:864894.
Yanez-Mo M, Siljander PR, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles. 2015;4:27066.
Yang X, Weng Z, Mendrick DL, et al. Circulating extracellular vesicles as a potential source of new biomarkers of drug-induced liver injury. Toxicol Lett. 2014;225:401–6.

Squamous Cell Carcinoma Antigen-Immunoglobulin M (SCCA-IgM) as Biomarker in Liver Disease: Biological Aspects and Clinical Applications

27

A. Biasiolo, A. Martini, A. Gallotta, G. Fassina, and P. Pontisso

Contents

Abstract

Chronic liver diseases and cirrhosis are an increasing cause of morbidity and mortality in Western countries. In particular, liver cirrhosis can be an asymptomatic and silent condition until clinical decompensation occurs, which could lead to organ failure, with a mortality rate up to 30%. One of the main causes of mortality in patients with cirrhosis is hepatocellular carcinoma, one of the most common fatal cancers worldwide, the 4th one for incidence rate. A high public health priority need is the development of biomarkers to screen for liver disease progression and for early diagnosis of hepatocellular carcinoma development, particularly in the high-risk population represented by patients with cirrhosis. Recently, circulating immune complex squamous cell carcinoma antigen-IgM

A. Biasiolo (✉) • A. Martini (✉) • P. Pontisso (✉)
Department of Medicine, University of Padua, Padua, Italy
e-mail: alessandra.biasiolo@unipd.it; andremartini86@gmail.com; patrizia@unipd.it

A. Gallotta (✉) • G. Fassina (✉)
Xeptagen SpA, VEGA Park, Venice, Italy
e-mail: gallotta@xeptagen.com; fassina@xeptagen.com

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_15

has shown the ability to identify patients with progressive liver disease and patients at higher risk of hepatocellular carcinoma development. In this chapter, we describe the biochemical and biological features of squamous cell carcinoma antigen and the role of natural IgM to form immune complexes with altered self-antigen during carcinogenesis; we also present the main clinical studies that have investigated this new circulating biomarker in terms of diagnostic and prognostic value in chronic liver disease, liver cirrhosis, and hepatocellular carcinoma.

Keywords

SCCA-IgM • Biomarker • Cirrhosis • Hepatocellular carcinoma • HCV • Treatment • Prognosis

List of Abbreviations

AFP	Alpha-fetoprotein
AFP-L3	*Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein
AUROC	Area under receiver operating characteristic
C1q	Complement component 1q
DCP	Des-gamma carboxy-prothrombin
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
EpCam	Epithelial cell adhesion molecule
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIF-2α	Hypoxia-inducible factor-2 alpha
IFN-γ	Interferon gamma
IL-6	Interleukin 6
MBL	Mannose-binding lectin
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NF-kB	Nuclear factor-kappa light-chain enhancer of activated B cell
NK	Natural killer
NPV	Negative predictive value
NR	Null responders
PEG-IFN	Pegylated-interferon
RAS gene	Rat sarcoma gene
RF	Rheumatoid factor
RSL	Reactive-site loop
SCCA1/Serpin B3	Serpin member gene 3
SCCA2/serpinB4	Serpin member gene 4
SCCA-IgM	Squamous cell carcinoma antigen-immunoglobulin M
sIgM	Secreted IgM
SLE	Systemic lupus erythematosus

SVR	Sustained virological response
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
US	Ultrasound

Key Facts of Biomarkers in Liver Disease

- Liver diseases are often asymptomatic until clinical decompensation of cirrhosis occurs; because of this, liver disorders are often undiagnosed.
- Liver cirrhosis is the final stage of chronic inflammation, cell necrosis, and regeneration of the liver due to different types of damage that eventually contribute to functional and structural alterations of the liver. Cirrhosis is the main risk factor of hepatocellular carcinoma, an increasing cause of morbidity and mortality worldwide.
- At present, noninvasive and reliable serological biomarkers for early identification and surveillance of patients with liver disease are still an unmet need in clinical practice. Liver ultrasound is recommended for hepatocellular carcinoma surveillance in patients with cirrhosis. Alpha-fetoprotein is the most widely used serum marker for hepatocellular carcinoma diagnosis and surveillance; however, this biomarker has been defined inadequate for surveillance by international guidelines.
- In recent years, relevant emphasis has been attributed to natural autoantibodies of the IgM class, which can bind different markers expressed on cancer cells and facilitate their elimination by the immune system. IgM-linked immune complexes with diagnostic value have been found recently in different human tumors, including liver cancer.
- Several studies found squamous cell carcinoma antigen-IgM immune complexes in the serum of patients with evolving liver disease or hepatocellular carcinoma. Squamous cell carcinoma antigen is a protease inhibitor that is not present in normal hepatocytes, but can be induced as a defense mechanism in the presence of inflammation or hypoxic conditions. This protein protects the cell from apoptotic death and promotes cell proliferation, but in chronic conditions, it favors neoplastic transformation.
- Squamous cell carcinoma antigen-IgM can be detected in the serum of the majority of patients with hepatocellular carcinoma and in patients with hepatitis C virus infection. In patients with cirrhosis, the levels and/or the progressive increase of this biomarker has been correlated to the risk of liver tumor development, while in patients with chronic liver disease, squamous cell carcinoma antigen-IgM reflects liver disease activity. High levels of this biomarker are frequently found in patients with liver fibrosis progression and steatohepatitis. In agreement with these findings, clearance of hepatitis C virus by antiviral therapy is associated with significant decrease of squamous cell carcinoma antigen-IgM, suggesting that the serological level of this biomarker may be a reliable independent prognostic factor of therapeutic effectiveness.

Definitions of Words and Terms

Alpha-fetoprotein	A fetal plasma protein that is normally present in the amniotic fluid of pregnant women. In adults it is abnormally present in the blood in some forms of cancer, including hepatocellular carcinoma.
Biomarker (short for biological marker)	A biological measure of a biological state. By definition, it is an objectively measurable indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention.
Cancer immunoediting	A dynamic process that includes immunosurveillance and tumor progression. It consists of three phases: *elimination*, *equilibrium*, and *escape*. The elimination phase corresponds to immunosurveillance, the equilibrium phase represents the process by which the immune system selects tumor cell variants with increasing capacities to survive immune attack, and the escape phase is the uncontrolled tumor expansion in the immunocompetent host.
Cirrhosis	A late stage of scarring (fibrosis) of the liver caused by many forms of liver diseases and conditions, such as hepatitis and chronic alcohol abuse.
Hepatitis C virus	RNA virus that belongs to the *Hepacivirus* genus, member of the Flaviviridae family. Based on genetic differences, the hepatitis C virus species is classified into seven genotypes (1–7) with several subtypes within every genotype, each one with different susceptibility to antiviral therapy.
Hepatocellular carcinoma	Malignant liver tumor that arises from hepatocytes and develops mainly within an established background of liver cirrhosis.
Nonalcoholic steatohepatitis	A disease that is histologically defined according to a score based on the addition of clarification/ballooning and a perisinusoidal fibrosis semiquantitation. A score of >3 is considered diagnostic for nonalcoholic steatohepatitis.
Null response (to pegylated-interferon/ribavirin)	When hepatitis C virus RNA declines <2 log 10 IU/ml from baseline at 12 weeks post therapy.

Sustained virological response	When hepatitis C virus RNA is undetectable using a highly sensitive assay 24 weeks after the end of treatment.

Introduction

Liver cirrhosis is the final stage of chronic inflammation, cell necrosis, and hepatocellular regeneration due to different types of damage that eventually contribute to functional and structural alterations of the liver (Tsochatzis et al. 2014). From an epidemiological point of view, liver cirrhosis is an increasing cause of morbidity and mortality in Western countries and it is one the most common cause of death in adults worldwide (Blachier et al. 2013). The main causes of cirrhosis in developed countries are infection with hepatitis C virus (HCV), alcohol abuse, and, increasingly, nonalcoholic liver disease (Tsochatzis et al. 2014). In sub-Saharan Africa and in most parts of Asia, infection with hepatitis B virus (HBV) represents the most common cause of cirrhosis. The prevalence of this advanced liver disease is difficult to assess and probably higher than reported, because the initial stages are asymptomatic until cirrhosis with clinical decompensation occurs; therefore, the disorder is often undiagnosed (Tsochatzis et al. 2014). Hepatocellular carcinoma, one of the main complications of cirrhosis (Cabibbo and Craxi 2010; Bolondi and Gramantieri 2011), and the leading cause of death among these patients, is the sixth most common neoplasm and the third most frequent cause of cancer death (Forner et al. 2012). Whereas the survival of patients with most malignancies has enhanced over the last decade, 5-year survival rate of patients with HCC has not improved sufficiently and remains less than 10%. The poor outcome of patients with HCC is related to the late detection of the cancer, with the majority of patients diagnosed at advanced stages of the disease (Lok et al. 2010). It has been demonstrated that HCC surveillance of the population at risk increases survival, because of detection of tumors amenable to curative therapies (McMahon et al. 2000; Trevisani et al. 2002; Zhang et al. 2004); because of this, surveillance is recommended by international guidelines (Bruix and Sherman 2005). At present, noninvasive and reliable biomarkers for early identification and surveillance of patients with advanced liver disease are still an unmet need in clinical practice. Alpha-fetoprotein (AFP) is the most widely used serum marker for HCC diagnosis and surveillance; however, not all HCCs secrete AFP (about 32–59% of patients with HCC have normal AFP levels). Furthermore, AFP may be elevated in patients with chronic liver disease in the absence of HCC, making this biomarker inadequate for surveillance (Lok et al. 2010). Indeed, Western guidelines consider AFP too inaccurate to screen patients at risk of HCC and have recommend the use of ultrasound (US) alone (Bruix and Sherman 2011; European Association For The Study Of The Liver

et al. 2012). However, US sensitivity depends on many factors, including the quality of the US machine, the experience of the examiner, and also the patient characteristics. In patients with liver cirrhosis, regenerative nodules may be hard to distinguish from HCC using US, and the sensitivity of this imaging technique to detect early HCC lies between 32% and 65% (Singal et al. 2009). For this reason, some authors, as well as guidelines from Eastern countries, suggest the use of AFP for HCC surveillance (Poon et al. 2009; Lee et al. 2013). From 1990s, especially in Japan, new biomarkers for HCC diagnosis and surveillance have been explored. Among these, des-gamma carboxy-prothrombin (DCP), an abnormal prothrombin protein, has been considered (Li et al. 2014), but the results indicate that its sensitivity is highly dependent on tumor size (Nakamura et al. 2006). The clinical utility of *Lens culinaris* agglutinin (LCA)-reactive fraction of Alpha-fetoprotein (AFP-L3) in early prediction of HCC development in patients with chronic HBV or HCV infection was also recently evaluated (Schütte et al. 2015). It was shown that several factors (gender, age, race, and presence of more advanced liver disease) are independent predictors of increased levels of this biomarker, which also lacks in sensitivity, specificity, and predictive values required for routine HCC surveillance (Sterling et al. 2012). Another biomarker that has been developed in recent years is osteopontin, a molecule expressed by transformed malignant cells, also evaluated for colon and pancreatic cancer. The majority of the studies analyzing osteopontin for the diagnosis of HCC were retrospective and included a range of 30–179 patients with HCC. The reported sensitivity of osteopontin for HCC was 86%, with a specificity of 86%, resulting in a diagnostic accuracy comparable to that of AFP. Further validation studies are needed to use this marker in daily clinical routine (Wan et al. 2014). On the basis of the above considerations, a reliable biomarker to complement US in detecting early HCC still represents a crucial unmet need. In recent years, relevant emphasis has been ascribed to poly-reactive natural autoantibodies of IgM class that can bind different markers expressed during cancer growth with low affinity and high avidity (Vollmers and Brändlein 2007). IgM-linked immune complexes with diagnostic value have been found recently in different human tumors, including colon (Castaldi et al. 2005) and prostate (Beneduce et al. 2007) cancer, and also in other pathologic conditions, such as Alzheimer's disease (Marcello et al. 2009). A complete publication list regarding biomarkers-IgM is presented in Table 1. For liver disease, the diagnostic value of squamous cell carcinoma antigen (SCCA)-IgM immune complex in serum has been demonstrated in several studies (Beneduce et al. 2005; Zuin et al. 2010). SCCA-IgM is a circulating immune complex composed of IgM bound to a cancer biomarker, namely, SCCA. The occurrence of biomarker-IgM immune complexes is supported by recent knowledge on cancer immunoediting (Dunn et al. 2002; Bhardwaj 2007; Swann and Smyth 2007) that considers natural IgMs as one of the most important players of the innate immune systems against infectious agents and tumor cell growth. Multivalent IgMs can bind a wide range of posttranscriptionally modified tumor antigens expressed on neoplastic cells, and this interaction triggers the intrinsic apoptotic pathway, leading to specific cancer cell death (Vollmers and Brändlein 2007). In the first section of this chapter, we will discuss the biochemical and biological features

Table 1 Publication list of biomarkers-IgM grouped by organ and marker

Liver	
AFP-IgM	Crescenzi M, et al. Anal Methods. 7:629–637. 2015
	Gallotta A, et al. Clin Lab Med. 32:33–45. 2012
	Gomiero C, et al. J Hepatol. 54:S382. 2011
	Jiang J, et al. Anticancer Res. 31:687–92. 2011
	Krygier R, et al. Exp Clin Hep. 7:44–48. 2011
	Stefaniuk P, et al. World J Gastroenterol. 16:418–24. 2010
	Stefaniuk P, et al. Exp Clin Hep. 6:AB18–18. 2010
	Teofanescu I, et al. Rev Med Chir Soc Med Nat Iasi. 114:39–46. 2010
	Gallotta A, et al. Int J Biol Markers. 24:208. 2009
	Jingting J, et al. J Clin Lab Anal. 23:213–8. 2009
	Kumar M, et al. Current Trends in Science. 403–417. 2009
	Sheng SL, et al. J Clin Lab Anal. 23:179–85. 2009.
	Giannelli G, et al. Recenti Prog Med. 98:23–8. 2007
	Giannelli G, et al. Clin Chim Acta. 383:147–52. 2007
	Beneduce L, et al. J Hepatol. 44:S97. 2006
	Beneduce L, et al. Int J Biol Markers. 19:155–9. 2004
DCP-IgM	Bertino G, et al. Minerva Med. 102:363–71. 2011
	Beneduce L, et al. Eur J Clin Invest. 38:571–7. 2008
	Beneduce L, et al. J Hepatol. 44:S96. 2006
MMP9-IgM	Agostini M, et al. Gut. 57:A360. 2008
SCCA-IgM	Crescenzi M, et al. Anal Methods. 7:629–637. 2015
	Liu J, et al. Arch. Med. Res. S0188–4409. 2015
	Martini A, et al. J Viral Hepat. 22(10):800–808. 2015
	Montagnana M, et al. Clin Chim Acta. 445:161–166. 2015
	Morisco F, et al. J Hepatol. 62:S450. 2015
	Tsuchiya N, et al. World J Gastroenterol. 21(37):10573–10583. 2015
	Zhang J, et al. Mol Clin Oncol. 3:1165–1171. 2015
	Biasiolo A, et al. J Hepatol. 60:S259. 2014
	Gallotta A, et al. In Sensors (pp. 85–88). Springer New York. 2014
	Martini A, et al. J Hepatol. 60:S350–S351. 2014
	Mossad NA, et al. Tumour Biol. 35:11559–64. 2014
	Pontisso P. Ann Hepatol. 13:722–727. 2014
	Pozzan C, et al. J Gastroenterol Hepatol. 29:1637–44. 2014
	Biasiolo A, et al. J Med Virol. 85:1005–8. 2013
	Morisco F, et al. Gastroenterology. 144:S–991. 2013
	Biasiolo A, et al. PLoS ONE. 7:e40658. 2012
	Buccione D, et al. OJGas. 2:56–61. 2012
	Fransvea E, et al. J Viral Hepat. 19:704–710. 2012
	Gallotta A, et al. Clin Lab Med. 32:33–45. 2012
	Bertino G, et al. Minerva Med. 102:363–71. 2011
	Plebani M, et al. Clin Chem Lab Med. 49:759–60. 2011
	Pozzan C, et al. J Hepatol. 54: S101. 2011

(*continued*)

Table 1 (continued)

	Schmilovitz-Weiss H, et al. Diagn Pathol. 6:121. 2011
	Giannini EG, et al. J Viral Hepat. 17:563–568. 2010
	Stefaniuk P, et al. World J Gastroenterol. 16:418–24. 2010
	Stefaniuk P, et al. Exp Clin Hep. 6:AB18–18. 2010
	Teofanescu I, et al. Rev Med Chir Soc Med Nat Iasi. 114:39–46. 2010
	Yim S, et al. Cancers (Basel). 2:809–23. 2010
	Zuin J, et al. Clin Chem Lab Med. 48:217–23. 2010
	Biasiolo A, et al. Int J Biol Markers. 24:205. 2009
	Carrara S, et al. Sens Actuators B Chem. 136:163–172. 2009
	Gallotta A, et al. Int J Biol Markers. 24:208. 2009
	Hayashi M, et al. Rare Tumors. 1:e21. 2009
	Kumar M, et al. Current Trends in Science. 403–417. 2009
	Lunardi F, et al. Eur Respir J. 54:E3092. 2009
	Trerotoli P, et al. Mol Cancer. 8:29. 2009
	Vidalino L, et al. Autoimmun Rev. 9:108–12. 2009
	Villano G, et al. J Hepatol. 50:S71. 2009
	Zuin J, et al. Int J Biol Markers. 24:209. 2009
	Beale G, et al. BMC Cancer. 8:200. 2008
	Biasiolo A, et al. J Viral Hepat. 15:246–9. 2008
	Biasiolo A, et al. Gut. 57:A147. 2008
	Cagol M, et al. Gut. 57:A113. 2008
	Hussein MM, et al. Indian J Cancer. 45:167–72. 2008
	Vidalino L, et al. Clin Exp Rheumatol. 26:S83. 2008
	Fassan M, et al. Tumori. 93:518–21. 2007
	Giannelli G, et al. Recenti Prog Med. 98:23–8. 2007
	Giannelli G, et al. Clin Chim Acta. 383:147–52. 2007
	Giannelli G, et al. Clin Exp Rheumatol. 25:794–5. 2007
	Parenti A, et al. Histol Histopathol. 22:989–95. 2007
	Beneduce L, et al. J Hepatol. 44:S97. 2006
	Giacometti C, et al. Mod Pathol. 19:166. 2006
	Pontisso P, et al. Int J Cancer. 119:735–40. 2006
	Quarta S, et al. J Hepatol. 44:S107. 2006
	Beneduce L, et al. J Hepatol. 42:89. 2005
	Beneduce L, et al. Cancer. 103:2558–65. 2005
	Giannelli G, et al. Int J Cancer. 116:579–83. 2005
	Giannelli G, et al. Int J Cancer. 117:506–9. 2005
	Beneduce L, et al. J Hepatol. 40:77. 2004
	Pontisso P, et al. Br J Cancer. 90:833–7. 2004
Survivin-IgM	Matteucci C, et al. Hepatol. Res. 44:1008. 2014
	Matteucci C, et al. Int J Biol Markers. 24:210. 2009
VEGF-IgM	Biasiolo A, et al. Gut. 57:A145. 2008

(*continued*)

Table 1 (continued)

Prostate	
PSA-IgM	Gallotta A, et al. Cancer Biomark. 13:227–34. 2013
	Goc S, et al. Dis. Markers. 35:847–855. 2013
	Zani D, et al. Urologia. 77:1–3. 2010
	Zani D, et al. Int J Biol Markers. 24:212. 2009
	Beneduce L, et al. Cancer Detect Prev. 31:402–7. 2007
	Beneduce L, et al. Eur Urol Suppl. 5:163. 2006
Colon	
CEA-IgM	Kojima T, et al. Ann Cancer Res Therap. 19:15–19. 2011
	Kojima T, et al. Ann Cancer Res Therap. 18:69–72. 2010
	Kojima T, et al. Mol Med Report. 2:477–80. 2009
	Castaldi F, et al. Int J Biol Markers. 20:204–8. 2005
Esophagus	
CEA-IgM	Zorzetto V, et al. Dig Liver Dis. 43:S156. 2011
SCCA-IgM	Zorzetto V, et al. Dig Liver Dis. 44:S151. 2012
	Zorzetto V, et al. Dig Liver Dis. 43:S156. 2011
Bone	
AFP-IgM	Savitskaya Y, et al. Biomark Cancer. 2:65–78. 2010
CEA-IgM	Savitskaya Y, et al. Biomark Cancer. 2:65–78. 2010
SCCA-IgM	Savitskaya Y, et al. Biomark Cancer. 2:65–78. 2010

of SCCA and the role of SCCA-IgM in immunological cancer control. The behavior and clinical significance of SCCA-IgM in serum in different stages of liver disease will be addressed in the second part, with particular attention to the prognostic value of the biomarker in clinical settings and in monitoring patients with chronic hepatitis C in relation to their response to antiviral treatment.

Squamous Cell Carcinoma Antigen (SCCA)

SCCA, with its two highly homologous isoforms SCCA1 (or SerpinB3) and SCCA2 (or SerpinB4), belongs to the serine protease inhibitors family, and it was originally purified from a squamous cell carcinoma of the uterine cervix (Kato and Torigoe 1977). Serpins are encoded by two separate genes located on chromosome 18q21.3; recently they've been known as serpin peptidase inhibitor clade B member gene 3 (SerpinB3) or serpin peptidase inhibitor clade B member gene 4 (SerpinB4) (Suminami et al. 1991). The genes of the two isoforms, which share a high degree of homology (up to 98%), encode for two glycoproteins with a molecular weight of 45 kDa, composed by 390 amino acids with up to 92% similar composition. SCCA1 and SCCA2 show distinct properties and substrate specificities. SCCA1/SerpinB3 inhibits papain-like cysteine proteases (Schick et al. 1998), whereas SCCA2/SerpinB4 inhibits both serine

and cysteine proteases (Schick et al. 1997). The specific function or target depends mainly on the variety of the reactive-site loop (RSL), which is involved in the interaction with the protease, its recognition, and cleavage, resulting in its inhibition (Masumoto et al. 2003; Sakata et al. 2004). Target specificity is the result of a difference in the serpin's RSL sequence, in which only 7 out of 13 amino acid residues (54%) are identical. SCCA is physiologically expressed in the basal and parabasal layers of normal squamous epithelium, in endothelial cells of the veins, in arterial walls (Turato et al. 2012), and in peripheral blood mononuclear cells (Chechlinska et al. 2010); also, the protein is overexpressed in neoplastic cells of epithelial origin (Takeshima et al. 1992; Kato 1996; Cataltepe et al. 2000). To assess the biological influence of the serpin in vivo, Villano et al. have recently evaluated its effect in a SCCA1/SerpinB3 transgenic mouse model; the results indicate that the serpin induced an increase of about 15% in survival length, compared to controls. This study suggests a protective effect of SCCA1/SerpinB3 "per se"; the protein could become harmful and favor tumor development only in the presence of additional hits, such as inflammation, as it occurs in the case of chronic liver damage (Villano et al. 2013). In the liver, SCCA isoforms are undetectable in normal hepatocytes, but their expression progressively increases across a continuum of precancerous diseases, from chronic liver disease (Beneduce et al. 2005) to dysplastic nodules (Guido et al. 2008) and HCC (Pontisso et al. 2004; Trerotoli et al. 2009), suggesting a possible involvement of SCCA from the early events of the complex process of hepatocarcinogenesis. Indeed, SCCA-1/SerpinB3 isoform has been found in hepatoblastoma, which is considered an embryonal tumor of the liver (Turato et al. 2012). More recently, its presence has been described in the hepatic epithelial cell adhesion molecule (EpCAM)-positive stem/progenitor cells, both in human fetal livers and in adult livers; these findings were corroborated by the induction of the serpin in a mouse model of liver stem/progenitor cell activation (Villano et al. 2014). Liver tumors with stemness signature are highly aggressive and along this line SCCA1/SerpinB3 has been found overexpressed, together with transforming growth factor-beta (TGF-β), in a subset of aggressive forms of primary liver cancer, characterized by early tumor recurrence (Turato et al. 2014). SCCA seems to provide cell resistance to apoptosis induced by different kinds of stimuli, such as radiation (Murakami et al. 2001), tumor necrosis factor-alpha (TNF-α) (Suminami et al. 2000), or chemotherapeutic agents (Ciscato et al. 2014). The pro-oncogenic potential of SCCA has been further explored in the last decade by both in vitro and in vivo experiments. At cellular level, Quarta et al. reported that SCCA1/SerpinB3 induces cell proliferation, associated with downregulation of E-cadherin, increased β-catenin expression, and deregulation of adhesion processes (decrease desmosomal junctions) – typical features of epithelial-mesenchymal transition (EMT), a process that promotes cellular invasion (Quarta et al. 2010). Features of EMT and increased invasiveness, determined by this serpin, are similar to those already reported for hypoxia (Cannito et al. 2008). Recent investigation has indeed shown that SCCA1/SerpinB3 is upregulated also in hypoxic conditions in liver cancer cells through a selective hypoxia-inducible factor 2 alpha (HIF-2 α)-dependent mechanism (Cannito et al. 2015). Other findings revealed that SCCA isoforms are rat sarcoma (RAS)-responsive factors, able to induce inflammatory protein production and tumorigenesis: Catanzaro et al. have documented a positive correlation

between RAS mutation and enhanced SCCA1/SerpinB3 and interleukin-6 (IL-6) expression in samples of human colorectal and pancreatic tumors, reflecting an inflammatory response related to the nuclear factor kappa-light chain enhancer of activated B cell (NF-kB) (Catanzaro et al. 2014).

Natural IgM in Cancer Immunoediting

Free SCCA is barely detectable in serum of patients with advanced liver disease and primary liver cancer, while SCCA-IgM complexes are abundant (Beneduce et al. 2005; Giannelli et al. 2007a). The occurrence of biomarker-IgM immune complexes is the result of cancer immunoediting: a process in which natural IgMs are important players of the innate immune system preventing tumor formation (Dunn et al. 2002). The secreted pentameric form of IgM (sIgM), beside its well-known role in recognition and elimination of external invaders such as bacteria and viruses, acts as natural IgM to remove apoptotic cells through the phagocytic process. Natural IgMs attach to apoptotic cells through N-glycans expressed in the IgM constant region and then recruit mannose-binding lectin (MBL). The complement component 1q (C1q) binds a central protruding region of natural IgMs to form the IgM-C1q-MBL complex, which is able to connect phagocytes to apoptotic cells, therefore promoting the clearance of the latter (Ehrenstein and Notley 2010) (Fig. 1). In addition to the removal of apoptotic cells, mounting evidence indicates that natural IgMs are also involved in the immune surveillance against precancerous and cancerous cells. Dunn et al. envisaged a model of cancer immunoediting as a result of three processes: elimination, equilibrium, and escape. In this model, the neo-epitopes expressed on the tumor cell surface are recognized by circulating sIgM, which enhance phagocytic clearance of transformed cell by macrophages and dendritic cells (Silverman 2011). Although little is known about the mechanism of IgM-mediated phagocytosis, this pathway is likely to reflect a host immune protective mechanisms that applies selective pressure on newly developed neoplastic cells (elimination phase, previously known as immune surveillance). When malignant cells develop a mechanism to escape the selective pressure of the immune system (escape phase), the tumor is free to continue growing. The model also includes a temporary, sometimes very long, equilibrium phase in which the components of the immune system (lymphocytes, IFN-γ, natural IgMs) exert a potent selective pressure on the neoplastic cells that is enough to contain, but not fully extinguish, the genetically unstable and rapidly mutating tumor cells (Fig. 2). According to the new theory on cancer immunoediting, the presence of circulating SCCA-IgM immune complexes reflects the efficient repair mechanisms of the innate immunity, in which natural IgM antibodies are involved in the recognition of new epitopes (e.g., carbohydrate residues) on posttranscriptionally modified cell surface receptors of precancerous and cancerous cells (Hensel et al. 1999). To date, there is no direct evidence for any structural change on SCCA in patients with chronic liver disease; however, posttranscriptional polymerization or glycosylation/fucosylation of the serpin during liver disease progression cannot be excluded.

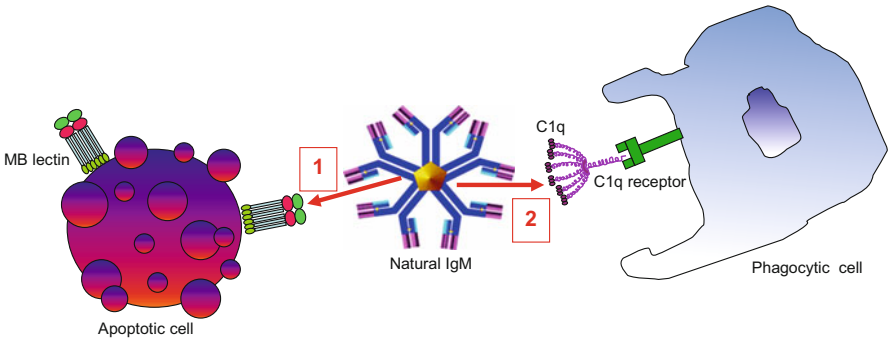

Fig. 1 Schematic representation of IgM-induced apoptotic cell clearance. Model of the proposed mechanism of natural IgM involvement in the induction of apoptotic cell death. Natural IgMs attach to apoptotic cells through N-glycans in the IgM constant region and recruit mannose-binding lectin (*MB lectin*). The complement component 1q (*C1q*) then binds a central protruding region of natural IgMs, and this IgM-C1q-MB lectin complex recruits phagocytes to apoptotic cells, favoring their clearance

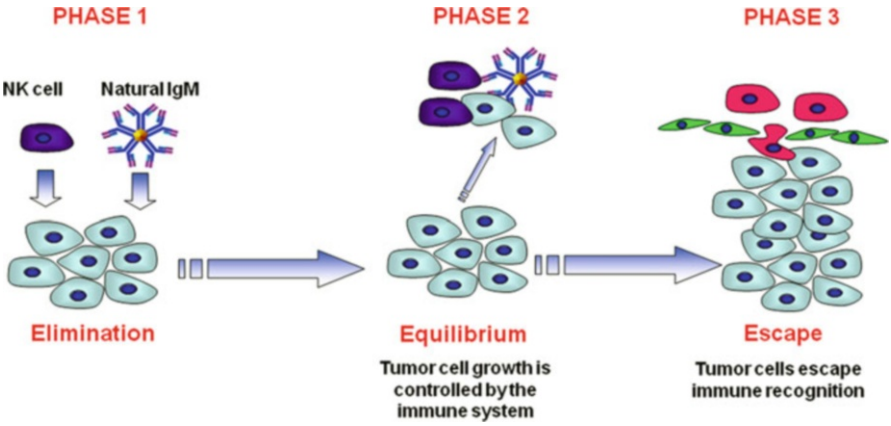

Fig. 2 The cancer immunoediting model. The proposed cancer immunoediting process is the result of three phases: elimination, equilibrium, and escape (three "E" processing). In this model, the neo-epitopes on tumor cells surface (*light blue*) are recognized by lymphocytes that participate in innate immunity, such as natural killer cells (*NK*), and by circulating IgMs that enhance apoptotic cells' phagocytic clearance. In the equilibrium phase, the tumor growth is controlled by the immune system, but when malignant cells (*pink*) develop mechanisms to escape the selective pressure of the immune system (Escape), the tumor can continue to grow

Clinical Studies on SCCA-IgM in Liver Diseases

The clinical usefulness of monitoring SCCA-IgM immune complexes in chronic liver disease has been evaluated in several studies. In a study published in 2008, SCCA-IgM was detected, at presentation, in 33% of untreated patients with

histologically proven chronic hepatitis, but not in healthy control subjects. After a median period of 6 years, an increased level of the immune complex was observed in 75% of cases with progressive disease confirmed by biopsy (defined as an increase in liver fibrosis score ≥ 2 during follow-up in untreated patients). On the other hand, SCCA-IgM levels were substantially stable in patients with no disease progression during the same interval, and no difference in the level of the biomarkers was detected in regard to the etiology of chronic liver disease (Biasiolo et al. 2008). In chronic HCV infection, the presence of nonalcoholic steatohepatitis (NASH) at the histological level reflects a more severe clinical and pathological state than steatosis alone, being associated with a more rapid progression of fibrosis (Bedossa et al. 2007). Recently, the relationship between SCCA-IgM and NASH was investigated in 91 patients with chronic hepatitis C: in those with histological diagnosis of NASH, the immune complex levels were elevated and associated with more severe steatosis (>33%). The association between SCCA-IgM and NASH in HCV positive patients was confirmed at univariate and multivariate logistic regression analysis. Among the various clinical aspects that were considered, only HCV genotype 3 was identified as an additional independent variable significantly associated with NASH (Martini et al. 2015). Furthermore, a close correlation between the intensity of SCCA-1 expression in the liver and serological SCCA-IgM levels was documented in serum and liver samples from the same patients: in cases of negative serological SCCA-IgM, SCCA-1 detection in the corresponding liver biopsy was weak, even in the presence of steatosis; on the other hand, the serpin was highly expressed in patients with elevated serum values of SCCA-IgM (Martini et al. 2015). Serological levels of IgM-linked SCCA isoforms have been evaluated in patients with different extent of chronic liver disease, and an isoform-specific immunoenzymatic assay using anti-SCCA1 and anti-SCCA2 specific monoclonal antibodies was set up. Although the number of patients was limited, this study revealed an altered balance of the two serpin isoforms in HCC; specifically, lower SCCA2-IgM levels and a progressive decrease of SCCA2-IgM/SCCA1-IgM median ratio in patients with more advanced liver disease (1.08 in patients with HCC, 1.10 in patients with cirrhosis, and 1.4 in patients with chronic hepatitis) (Biasiolo et al. 2012). Like most of the circulating immune complexes, SCCA-IgM is usually detected in serum by means of ELISA, which might be invalidated by the presence of endogenous immunoglobulins such as IgM with rheumatoid factor (RF) activity. For this reason, the specificity of SCCA-IgM in relation to the presence of RF activity was investigated in 73 patients with cirrhosis and infected with HCV. Patients with RF activity had significantly higher levels of SCCA-IgM compared to RF-negative cases (median 702 AU/ml vs. 131 AU/ml; $p < 0.0001$). This finding was not surprising, because HCV, by means of CD81 receptor located on hepatocytes and B lymphocytes surface, can determine stimulatory signals that lower the threshold required for B cells to respond to external antigens. This interaction determines an efficient polyclonal expansion of autoreactive clones that can be readily activated to produce antibodies, including natural IgMs. To exclude any possible unspecific interference of RF in the SCCA-IgM assay, the standard positive calibrator was spiked with serial dilutions of RF-positive or RF-negative serum. In these artificially created samples,

similar results in terms of reactivity for SCCA-IgM were obtained, regardless of the presence of RF activity (Biasiolo et al. 2013).

SCCA-IgM Behavior During Antiviral Treatment

Combination therapy of pegylated-interferon-alpha (PEG-IFN) and ribavirin results in complete viral eradication in about 50% of patients with chronic HCV infection. However, a substantial number of patients show no significant response to therapy or develop viral relapse after the cessation of IFN-based therapy (McHutchison et al. 2009). The first evidence of the behavior of SCCA-IgM during antiviral treatment with PEG-IFN and ribavirin was obtained from a longitudinal study in 2010. Giannini et al. demonstrated that in patients with HCV-related cirrhosis who achieved sustained virological response (SVR) there was a significant decrease in serum levels of SCCA-IgM at the end of treatment, and up to 1 year of follow-up, when compared to baseline. In null responders (NR), baseline values of serum SCCA-IgM were not statistically different from SVR patients, but during follow-up, serological SCCA-IgM levels did not show significant changes compared to baseline (Giannini et al. 2010). In 2012, in a multicentre prospective study, 103 patients with HCV chronic infection undergoing antiviral treatment with PEG-IFN and ribavirin were enrolled to test the efficacy of SCCA-IgM as a marker of response (Fransvea et al. 2012). This study confirmed that the reduction of SCCA immune complexes was significantly different between patients that showed SVR and those who did not. Moreover, the decreased serological concentration of the biomarker was an independent predictor of SVR in regard to age and HCV genotype. The behavior of SCCA-IgM in relation to antiviral therapy was recently confirmed in another study involving 91 patients with chronic hepatitis C. In the subgroup of patients who reached SVR and had a baseline positivity to SCCA-IgM, the serological values significantly decreased after 6 months of treatment and remained persistently low even at 6 months of follow-up after treatment. In NR patients, no significant variation in SCCA-IgM serum values was observed at the same time points (Martini et al. 2015). These studies clearly demonstrate that the termination of HCV-associated liver damage determines a progressive decline of SCCA-IgM serological levels (Fig. 3), therefore this biomarker could be used as a surrogate marker to monitor active disease resolution.

SCCA-IgM and HCC

One of the most important and yet unmet clinical needs in hepatology is the availability of serological markers to identify patients with cirrhosis at higher risk of HCC development. The incidence of hepatocellular carcinoma in individuals with HCV cirrhosis is 3–5% per year (Fattovich et al. 1997); the identification of the subgroup of patients with possible HCC development within the next few years would allow the development of a personalized clinical management characterized

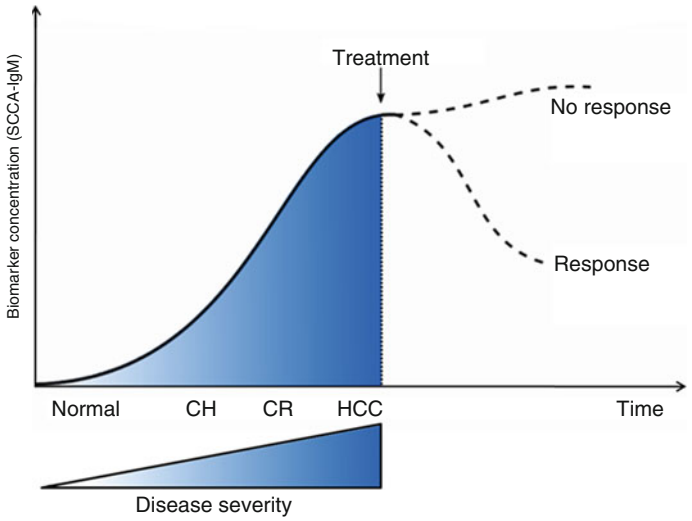

Fig. 3 Monitoring liver disease progression by SCCA-IgM. Schematic representation of changes in serological SCCA-IgM levels in different stages of chronic liver disease. *CH* chronic hepatitis, *CR* cirrhosis, *HCC* hepatocellular carcinoma

by more effective early therapeutic interventions. In order to explore this possibility, SCCA-IgM was analyzed in a retrospective, longitudinal study that was preliminary conducted in a cohort of HCV-infected patients with early stage of cirrhosis, defined on the basis of histological findings (Pontisso et al. 2006). The study population was divided into two groups with similar clinical characteristics and no significant difference in the absolute value of the immune complex at baseline. The first group included 16 cirrhotic patients who developed HCC during a median follow-up of 4 years, while the second group included 17 control patients with cirrhosis who did not develop HCC during the same period. The progressive increase of SCCA-IgM over time was remarkable in cirrhotic patients who eventually developed HCC, while figures remained unchanged or decreased in the majority of the cirrhotic patients without evidence of HCC during the same time interval. Conversely to SCCA-IgM, AFP increase was not significantly different in the two groups of cirrhotic patients (Pontisso et al. 2006).

These data were in line with another retrospective study performed by Buccione et al. The aim of this study was to evaluate whether the levels of SCCA-IgM in serum could identify HCV patients with clinical signs of cirrhosis at risk of HCC development. The study involved 57 cirrhotic patients, during a median period of 48 months. The baseline value of serological SCCA-IgM was nearly fourfold higher in patients who developed HCC than in those who did not, and the SCCA-IgM value $\leq$200 AU/mL accurately identified patients at low risk of liver cancer in the subsequent year, with a negative predictive value (NPV) of 97% (Buccione et al. 2012). These results suggest that in patients with overt cirrhosis the assessment of this biomarker could improve the diagnostic process, since the subgroup of

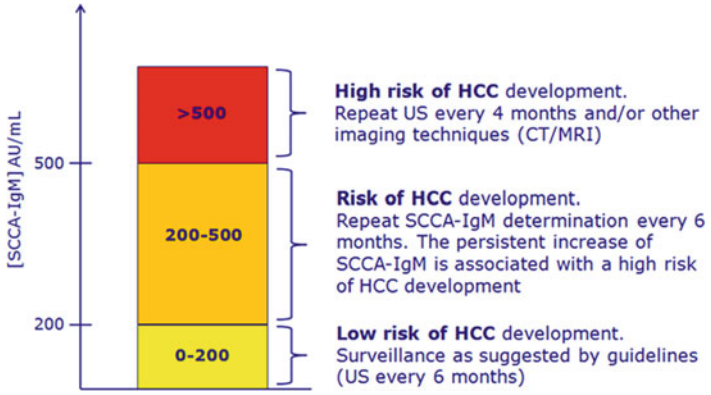

Fig. 4 Serological surveillance with SCCA-IgM. Model of serological surveillance with an annual analysis of SCCA-IgM levels in patients with cirrhosis who are negative at the first US analysis

patients at higher risk of liver tumor development and who need a constant monitoring could be identified based on SCCA-IgM positivity (Fig. 4). In regard to the diagnostic value of SCCA-IgM for HCC, a cross-sectional study performed by Beneduce et al. has demonstrated the positivity of this biomarker in the vast majority of HCC serum samples (70% sensitivity vs. 42% sensitivity of AFP), whereas all healthy control samples were negative. The authors observed that circulating SCCA-IgM at different stages of liver disease (positivity: 18% in chronic hepatitis, 26% in cirrhosis, and 70% in HCC) reflected the extent of SCCA expression detected by immunohistochemistry in liver specimens. Moreover, SCCA-IgM positivity did not overlap with that of AFP, suggesting that the combination of these two biomarkers could improve the diagnostic sensitivity for detecting HCC without compromising the diagnostic specificity (Beneduce et al. 2005). Mossad et al. in a smaller cross-sectional study involving 40 patients with HCC, 30 with liver cirrhosis, and 20 healthy controls compared the diagnostic accuracy of SCCA-IgM, alpha-L-fucosidase (AFU), and AFP in early diagnosis of HCC. The diagnostic performance of SCCA-IgM (sensitivity 88%, specificity 66%) was found to be higher than that AFP (sensitivity 70%, specificity 53%). Again, the combined measurement of both biomarkers increased the diagnostic sensitivity (93%), while the specificity remained substantially unchanged (62%) (Mossad et al. 2014). In a large study including 499 HCC and 462 cirrhotic patients, Giannelli et al. have compared free SCCA, free AFP, SCCA-IgM, and AFP-IgM (Giannelli et al. 2007a). AFP showed the best performance with an AUROC of 0.724 (sensitivity 41%, specificity 94%), while for AFP-IgM AUROC value was 0.667 (sensitivity 39%, specificity 91%). The diagnostic performance of serum SCCA-IgM (AUROC 0.675; sensitivity 52%, specificity 76%) was slightly higher than that of free SCCA (AUROC 0.656; sensitivity 42%, specificity 83%). No significant correlation was found between AFP, AFP-IgM, SCCA-IgM, and tumor size, while SCCA levels were significantly increased ($p < 0.0001$) in smaller nodules (<3 cm): a characteristic that suggests a

possible use of this biomarker to detect HCC at its early onset. The combination of AFP (cut-off value: 21 IU/ml) and SCCA-IgM (cut-off value: 104 AU/ml) confirmed the diagnosis of HCC in 54% of the patients.

Until recently no data were available on the prognostic role of SCCA-IgM in HCC prognosis. This aspect was addressed in a recent study by Pozzan et al. who retrospectively analyzed the serum of 327 patients with cirrhosis and HCC. The ability of SCCA-IgM to predict HCC prognosis was proved for the first time: low concentration of this biomarker identified HCC patients with longer overall and progression-free survival. Median survival was 48 months for patients with low SCCA-IgM (<130 AU/ml) and 26 months for those with high SCCA-IgM (>130 AU/ml). The levels of the biomarker at 4 weeks were stable or increased in treated patients with stable disease or tumor and reduced in patients with complete response, while patients with partial response showed an intermediate behavior. In the same study, AFP was not able to predict complete response. The significant impact of SCCA-IgM determination in defining patient prognosis was confirmed also by data showing that SCCA-IgM levels and tumor size were the only identified independent predictors of overall survival (Pozzan et al. 2014). Although these findings must be confirmed in further studies, they are supported by recent data demonstrating that liver tumors with high SCCA-1 tissue expression exhibit higher early recurrence after surgical resection (Turato et al. 2014).

In summary, the prognostic role of SCCA-IgM has been explored both in patients with chronic hepatitis and with cirrhosis, documenting a higher risk of fibrosis progression and of liver tumor development, respectively (Figs. 3 and 5).

Potential Applications to Other Diseases or Conditions

Alteration in SCCA function is associated with deregulation of cell survival and with some autoimmune traits indeed; people carrying serpin dysfunction often display an altered immune response. SCCA was found to be absent in autoimmune diseases such as systemic lupus erythematosus (SLE) CD27+ B lymphocytes, consistent with its expression being suppressed by high levels of type I interferon, which is a typical finding in SLE (Vidalino et al. 2012). Overexpression of SCCA has been reported in lung tissue of patients with idiopathic pulmonary fibrosis, but not in other forms of interstitial lung disease or normal lungs (Calabrese et al. 2008). Moreover, mice transgenic for human SERPINB3 showed higher TGF-β expression and more extended pulmonary fibrosis than controls (Lunardi et al. 2011). In keeping with the above findings, it has been reported that SCCA-IgM is increased in scleroderma patients with lung fibrosis (Giannelli et al. 2007b). The protective role of SCCA isoforms has been documented in patients affected by diabetic foot ulcers: a study aimed to detect candidate targets to distinguish patients with rapid healing compared with nonhealing wounds demonstrated that the SCCA/total protein ratio was 2.12-fold higher in patients who improved compared to those who did not. These results suggest that SCCA can be considered as a biomarker of successful healing, and it could be employed clinically to stratify the probability of good versus bad wound outcome (Fadini et al. 2014).

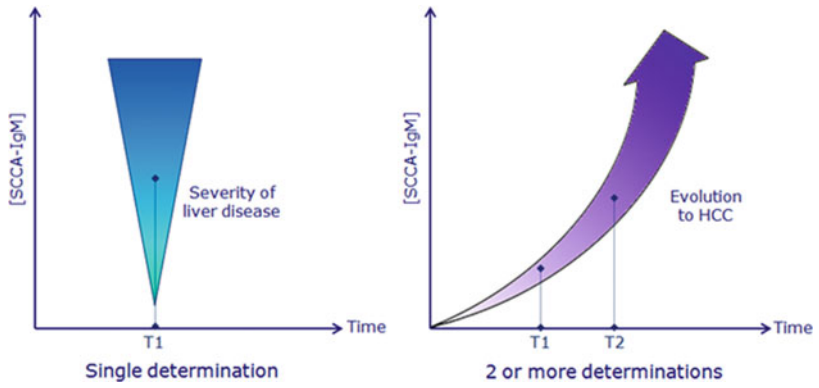

Fig. 5 SCCA-IgM clinical application. *Left* A single determination of SCCA-IgM can be useful to determine the severity of liver disease. *Right* Serial determinations of SCCA-IgM may indicate evolution of the disease to more severe forms (e.g., from hepatitis to cirrhosis, to HCC)

Summary Points

- This chapter focuses on squamous cell carcinoma antigen-IgM, which is a circulating immune complex composed of IgM bound to the liver biomarker squamous cell carcinoma antigen.
- The presence of circulating squamous cell carcinoma antigen-IgM likely reflects the efficient repair mechanisms of the innate immunity to prevent tumor formation.
- The clinical usefulness of monitoring squamous cell carcinoma antigen-IgM immune complexes in chronic liver disease has been evaluated in several studies.
- Squamous cell carcinoma antigen-IgM is undetectable in the serum of healthy subjects, but in chronic hepatitis, cirrhosis, and hepatocellular carcinoma, the detection rates increase consistently with liver disease progression.
- Squamous cell carcinoma antigen-IgM is a novel biomarker that can identify patients with progressive liver disease and patients at higher risk of HCC development.
- Monitoring of SCCA-IgM behavior during treatment of chronic hepatitis C could be useful to examine the response to antiviral therapy.

References

Bedossa P, Moucari R, Chelbi E, et al. Evidence for a role of nonalcoholic steatohepatitis in hepatitis C: a prospective study. Hepatology. 2007;46:380–7.

Beneduce L, Castaldi F, Marino M, et al. Squamous cell carcinoma antigen-immunoglobulin M complexes as novel biomarkers for hepatocellular carcinoma. Cancer. 2005;103:2558–65.

Beneduce L, Prayer-Galetti T, Giustinian AM, et al. Detection of prostate-specific antigen coupled to immunoglobulin M in prostate cancer patients. Cancer Detect Prev. 2007;31:402–7.
Bhardwaj N. Harnessing the immune system to treat cancer. J Clin Invest. 2007;117:1130–6.
Biasiolo A, Chemello L, Quarta S, et al. Monitoring SCCA-IgM complexes in serum predicts liver disease progression in patients with chronic hepatitis. J Viral Hepat. 2008;15:246–9.
Biasiolo A, Tono N, Ruvoletto M, et al. IgM-linked SerpinB3 and SerpinB4 in sera of patients with chronic liver disease. PLoS One. 2012;7:e40658.
Biasiolo A, Tono N, Zaninotto M, et al. Specificity of squamous cell carcinoma antigen (SCCA)-IgM detection in patients with HCV infection and rheumatoid factor seropositivity. J Med Virol. 2013;85:1005–8.
Blachier M, Leleu H, Peck-Radosavljevic M, et al. The burden of liver disease in Europe: a review of available epidemiological data. J Hepatol. 2013;58:593–608.
Bolondi L, Gramantieri L. From liver cirrhosis to HCC. Intern Emerg Med. 2011;6 Suppl 1:93–8.
Bruix J, Sherman M, Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. Hepatology. 2005;42:1208–36.
Bruix J, Sherman M, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. Hepatology. 2011;53:1020–2.
Buccione D, Fatti G, Gallotta A, et al. Serum SCCA-IgM as a predictor of hepatocellular carcinoma in patients with liver cirrhosis. Open J Gastroenterol. 2012;2:56–61.
Cabibbo G, Craxi A. Epidemiology, risk factors and surveillance of hepatocellular carcinoma. Eur Rev Med Pharmacol Sci. 2010;14:352–5.
Calabrese F, Lunardi F, Giacometti C, et al. Overexpression of squamous cell carcinoma antigen in idiopathic pulmonary fibrosis: clinicopathological correlations. Thorax. 2008;63:795–802.
Cannito S, Novo E, Compagnone A, et al. Redox mechanisms switch on hypoxia-dependent epithelial-mesenchymal transition in cancer cells. Carcinogenesis. 2008;29:2267–78.
Cannito S, Turato C, Paternostro C, et al. Hypoxia up-regulates SERPINB3 through HIF-2alpha in human liver cancer cells. Oncotarget. 2015;6:2206–21.
Castaldi F, Marino M, Beneduce L, et al. Detection of circulating CEA-IgM complexes in early stage colorectal cancer. Int J Biol Markers. 2005;20:204–8.
Cataltepe S, Gornstein ER, Schick C, et al. Co-expression of the squamous cell carcinoma antigens 1 and 2 in normal adult human tissues and squamous cell carcinomas. J Histochem Cytochem. 2000;48:113–22.
Catanzaro JM, Sheshadri N, Pan JA, et al. Oncogenic Ras induces inflammatory cytokine production by upregulating the squamous cell carcinoma antigens SerpinB3/B4. Nat Commun. 2014;5:3729.
Chechlinska M, Kowalewska M, Brzoska-Wojtowicz E, et al. Squamous cell carcinoma antigen 1 and 2 expression in cultured normal peripheral blood mononuclear cells and in vulvar squamous cell carcinoma. Tumour Biol. 2010;31:559–67.
Ciscato F, Sciacovelli M, Villano G, et al. SERPINB3 protects from oxidative damage by chemotherapeutics through inhibition of mitochondrial respiratory complex I. Oncotarget. 2014;5:2418–27.
Dunn GP, Bruce AT, Ikeda H, et al. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3:991–8.
Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. Nat Rev Immunol. 2010;10:778–86.
European Association For The Study Of The Liver, European Organisation For Research And Treatment Of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol. 2012;56:908–43.
Fadini GP, Albiero M, Millioni R, et al. The molecular signature of impaired diabetic wound healing identifies serpinB3 as a healing biomarker. Diabetologia. 2014;57:1947–56.
Fattovich G, Giustina G, Degos F, et al. Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. Gastroenterology. 1997;112:463–72.
Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet. 2012;379:1245–55.

Fransvea E, Trerotoli P, Sacco R, et al. SCCA-IC serum levels are predictive of clinical response in HCV chronic hepatitis to antiviral therapy: a multicentric prospective study. J Viral Hepat. 2012;19:704–10.

Giannelli G, Fransvea E, Trerotoli P, et al. Clinical validation of combined serological biomarkers for improved hepatocellular carcinoma diagnosis in 961 patients. Clin Chim Acta. 2007a;383:147–52.

Giannelli G, Iannone F, Fransvea E, et al. Squamous cellular carcinoma immunocomplexed is increased in scleroderma patients with lung fibrosis. Clin Exp Rheumatol. 2007b;25:794–5.

Giannini EG, Basso M, Bazzica M, et al. Successful antiviral therapy determines a significant decrease in squamous cell carcinoma antigen-associated (SCCA) variants' serum levels in anti-HCV positive cirrhotic patients. J Viral Hepat. 2010;17:563–8.

Guido M, Roskams T, Pontisso P, et al. Squamous cell carcinoma antigen in human liver carcinogenesis. J Clin Pathol. 2008;61:445–7.

Hensel F, Hermann R, Schubert C, et al. Characterization of glycosylphosphatidylinositol-linked molecule CD55/decay-accelerating factor as the receptor for antibody SC-1-induced apoptosis. Cancer Res. 1999;59:5299–306.

Kato H. Expression and function of squamous cell carcinoma antigen. Anticancer Res. 1996;16:2149–53.

Kato H, Torigoe T. Radioimmunoassay for tumor antigen of human cervical squamous cell carcinoma. Cancer. 1977;40:1621–8.

Lee E, Edward S, Singal AG, et al. Improving screening for hepatocellular carcinoma by incorporating data on levels of alpha-fetoprotein, over time. Clin Gastroenterol Hepatol. 2013;11:437–40.

Li C, Zhang Z, Zhang P, Liu J. Diagnostic accuracy of des-gamma-carboxy prothrombin versus alpha-fetoprotein for hepatocellular carcinoma: a systematic review. Hepatol Res. 2014;44: E11–25.

Lok AS, Sterling RK, Everhart JE, et al. Des-gamma-carboxy prothrombin and alpha-fetoprotein as biomarkers for the early detection of hepatocellular carcinoma. Gastroenterology. 2010;138:493–502.

Lunardi F, Villano G, Perissinotto E, et al. Overexpression of SERPIN B3 promotes epithelial proliferation and lung fibrosis in mice. Lab Invest. 2011;91:945–54.

Marcello A, Wirths O, Schneider-Axmann T, et al. Circulating immune complexes of Abeta and IgM in plasma of patients with Alzheimer's disease. J Neural Transm. 2009;116:913–20.

Martini A, Fattovich G, Guido M, et al. HCV genotype 3 and squamous cell carcinoma antigen (SCCA)-IgM are independently associated with histological features of NASH in HCV-infected patients. J Viral Hepat. 2015;22:800–8.

Masumoto K, Sakata Y, Arima K, et al. Inhibitory mechanism of a cross-class serpin, the squamous cell carcinoma antigen 1. J Biol Chem. 2003;278:45296–304.

McHutchison JG, Lawitz EJ, Shiffman ML, et al. Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. N Engl J Med. 2009;361:580–93.

McMahon BJ, Bulkow L, Harpster A, et al. Screening for hepatocellular carcinoma in Alaska natives infected with chronic hepatitis B: a 16-year population-based study. Hepatology. 2000;32:842–6.

Mossad NA, Mahmoud EH, Osman EA, et al. Evaluation of squamous cell carcinoma antigen-immunoglobulin M complex (SCCA-IGM) and alpha-L-fucosidase (AFU) as novel diagnostic biomarkers for hepatocellular carcinoma. Tumour Biol. 2014;35:11559–64.

Murakami A, Suminami Y, Hirakawa H, et al. Squamous cell carcinoma antigen suppresses radiation-induced cell death. Br J Cancer. 2001;84:851–8.

Nakamura S, Nouso K, Sakaguchi K, et al. Sensitivity and specificity of des-gamma-carboxy prothrombin for diagnosis of patients with hepatocellular carcinomas varies according to tumor size. Am J Gastroenterol. 2006;101:2038–43.

Pontisso P, Calabrese F, Benvegnu L, et al. Overexpression of squamous cell carcinoma antigen variants in hepatocellular carcinoma. Br J Cancer. 2004;90:833–7.

Pontisso P, Quarta S, Caberlotto C, et al. Progressive increase of SCCA-IgM immune complexes in cirrhotic patients is associated with development of hepatocellular carcinoma. Int J Cancer. 2006;119:735–40.
Poon D, Anderson BO, Chen LT, et al. Management of hepatocellular carcinoma in Asia: consensus statement from the Asian Oncology Summit 2009. Lancet Oncol. 2009;10:1111–8.
Pozzan C, Cardin R, Piciocchi M, et al. Diagnostic and prognostic role of SCCA-IgM serum levels in hepatocellular carcinoma (HCC). J Gastroenterol Hepatol. 2014;29:1637–44.
Quarta S, Vidalino L, Turato C, et al. SERPINB3 induces epithelial-mesenchymal transition. J Pathol. 2010;221:343–56.
Sakata Y, Arima K, Takai T, et al. The squamous cell carcinoma antigen 2 inhibits the cysteine proteinase activity of a major mite allergen, Der p 1. J Biol Chem. 2004;279:5081–7.
Schick C, Kamachi Y, Bartuski AJ, et al. Squamous cell carcinoma antigen 2 is a novel serpin that inhibits the chymotrypsin-like proteinases cathepsin G and mast cell chymase. J Biol Chem. 1997;272:1849–55.
Schick C, Pemberton PA, Shi GP, et al. Cross-class inhibition of the cysteine proteinases cathepsins K, L, and S by the serpin squamous cell carcinoma antigen 1: a kinetic analysis. Biochemistry. 1998;37:5258–66.
Schütte K, Schulz C, Link A, et al. Current biomarkers for hepatocellular carcinoma: surveillance, diagnosis and prediction of prognosis. World J Hepatol. 2015;7:139–49.
Silverman GJ. Regulatory natural autoantibodies to apoptotic cells: pallbearers and protectors. Arthritis Rheum. 2011;63:597–602.
Singal A, Volk ML, Waljee A, et al. Meta-analysis: surveillance with ultrasound for early-stage hepatocellular carcinoma in patients with cirrhosis. Aliment Pharmacol Ther. 2009; 30:37–47.
Sterling RK, Wright EC, Morgan TR, et al. Frequency of elevated hepatocellular carcinoma (HCC) biomarkers in patients with advanced hepatitis C. Am J Gastroenterol. 2012;107:64–74.
Suminami Y, Kishi F, Sekiguchi K, et al. Squamous cell carcinoma antigen is a new member of the serine protease inhibitors. Biochem Biophys Res Commun. 1991;181:51–8.
Suminami Y, Nagashima S, Vujanovic NL, et al. Inhibition of apoptosis in human tumour cells by the tumour-associated serpin, SCC antigen-1. Br J Cancer. 2000;82:981–9.
Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Invest. 2007;117:1137–46.
Takeshima N, Suminami Y, Takeda O, et al. Expression of mRNA of SCC antigen in squamous cells. Tumour Biol. 1992;13:338–42.
Trerotoli P, Fransvea E, Angelotti U, et al. Tissue expression of Squamous Cellular Carcinoma Antigen (SCCA) is inversely correlated to tumor size in HCC. Mol Cancer. 2009;8:29.
Trevisani F, De Notariis S, Rapaccini G, et al. Semiannual and annual surveillance of cirrhotic patients for hepatocellular carcinoma: effects on cancer stage and patient survival (Italian experience). Am J Gastroenterol. 2002;97:734–44.
Tsochatzis EA, Bosch J, Burroughs AK. Liver cirrhosis. Lancet. 2014;383:1749–61.
Turato C, Buendia MA, Fabre M, et al. Over-expression of SERPINB3 in hepatoblastoma: a possible insight into the genesis of this tumour? Eur J Cancer. 2012;48:1219–26.
Turato C, Vitale A, Fasolato S, et al. SERPINB3 is associated with TGF-beta1 and cytoplasmic beta-catenin expression in hepatocellular carcinomas with poor prognosis. Br J Cancer. 2014;110:2708–15.
Vidalino L, Doria A, Quarta S, et al. SerpinB3 expression on B-cell surface in autoimmune diseases and hepatitis C virus-related chronic liver infection. Exp Biol Med. 2012;237:793–802.
Villano G, Ruvoletto M, Ceolotto G, et al. SERPINB3 is associated with longer survival in transgenic mice. Sci Rep. 2013;3:3056.
Villano G, Turato C, Quarta S, et al. Hepatic progenitor cells express SerpinB3. BMC Cell Biol. 2014;15:5.
Vollmers HP, Brändlein S. Natural antibodies and cancer. J Autoimmun. 2007;29:295–302.
Wan HG, Xu H, Gu YM, et al. Comparison osteopontin vs AFP for the diagnosis of HCC: a meta-analysis. Clin Res Hepatol Gastroenterol. 2014;38:706–14.

Zhang BH, Yang BH, Tang ZY. Randomized controlled trial of screening for hepatocellular carcinoma. J Cancer Res Clin Oncol. 2004;130:417–22.

Zuin J, Veggiani G, Pengo P, et al. Experimental validation of specificity of the squamous cell carcinoma antigen-immunoglobulin M (SCCA-IgM) assay in patients with cirrhosis. Clin Chem Lab Med. 2010;48:217–23.

Peripheral Venous, Portal Venous, Hepatic Venous, and Arterial and Intrahepatic Cytokine Levels as Biomarkers and Functional Correlations

28

Wim Verlinden, Sven Francque, and Luisa Vonghia

Contents

Abstract

Measurements of cytokine levels in peripheral blood are frequently used to assess pathophysiological mechanisms in several liver diseases. The liver has a unique vasculature involving the portal vein, hepatic artery, and hepatic vein. It is strategically located between the digestive system and the systemic circulation and has a unique metabolic activity in terms of the processes of degradation and synthesis. The liver can both produce and clear cytokines. Therefore, one should

W. Verlinden (✉) • S. Francque (✉)
Department of Gastroenterology and Hepatology, Antwerp University Hospital, Edegem, Belgium

Laboratory of Experimental Medicine and Pediatrics, University of Antwerp, Wilrijk, Belgium
e-mail: Wim.Verlinden@uza.be; wimfmverlinden@gmail.com; Sven.Francque@uza.be

L. Vonghia (✉)
Department of Gastroenterology and Hepatology, Antwerp University Hospital, Edegem, Belgium
e-mail: Luisa.Vonghia@uza.be

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_22

consider the possibility that cytokines measured in peripheral blood do not always necessarily represent the cytokine profile present in hepatic or portal blood, or in liver tissue. Due to practical and ethical limitations, human studies evaluating the cytokine levels in different blood compartments are relatively rare. The studies reviewed in this chapter show us that the relationship or correlation between portal venous blood, hepatic venous blood, systemic venous blood, and arterial blood is not always present, nor is there per se a direct correlation between cytokine levels in blood compartments and the intrahepatic cytokine profile. The studies illustrate to us that this relationship can depend on the type of cytokine, presence of liver disease, disease severity, portal pressure, and/or liver function. Based on these studies, when studying the hepatic cytokine profile, one should not simply extrapolate the cytokine presentation in blood to the situation in liver tissue.

Keywords

Cytokines • Intrahepatic • Portal • Peripheral • Hepatic venous • Liver • Compartments

List of Abbreviations

AIH	Autoimmune hepatitis
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CD	Cluster of differentiation
CHB	Chronic viral hepatitis B
CHC	Chronic viral hepatitis C
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HVS	Hepatic venous system
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
L	Ligand
MCP	Macrophage chemoattractant protein
MDA	Malondialdehyde
mRNA	Messenger ribonucleic acid
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
PAI	Plasminogen activator inhibitor
PBC	Primary biliary cirrhosis
PBMC	Peripheral blood mononuclear cells

PVS	Portal venous system
s	Soluble
SVS	Systemic/peripheral venous system
TGF	Transforming growth factor
TIPS	Transjugular intrahepatic portosystemic shunt
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Key Facts of Transjugular Liver Catheterization

- A transjugular liver biopsy was first performed in 1967 and consists of obtaining liver tissue through a catheter introduced into one of the hepatic veins using jugular venous access.
- When there is a contraindication for transcutaneous intercostal liver biopsy, a transjugular liver biopsy can be an alternative approach.
- Transjugular catheterization can be used to determine the presence of portal hypertension, by measuring the difference between wedged and free hepatic venous pressure.
- Transjugular intrahepatic portosystemic shunt or transjugular intrahepatic portosystemic stent shunting (commonly abbreviated as TIPS or TIPSS) is performed by creating an artificial channel within the liver that establishes a direct communication between the inflow portal vein and the outflow hepatic vein.
- TIPS was first performed in 1982 to treat portal hypertension and its consequences (e.g., ascites and esophageal varices).

Definitions of Words and Terms

Adipokine	Adipokines are cytokines released by adipose tissue.
Antecubital vein	It is a superficial subcutaneous vein of the arm, which is also called the cephalic vein.
Bariatric surgery	Weight loss surgery includes a variety of procedures performed on people suffering from obesity. The surgery induces weight loss by changing the digestive system's anatomy, limiting the amount of food that can be eaten and/or digested.
Child-Pugh score	This score, sometimes called the Child-Turcotte-Pugh score is used to assess the prognosis of chronic liver diseases. It is based on the albumin and bilirubin level, prothrombin time, and the presence and severity of ascites and encephalopathy. Child-Pugh score is expressed as A, B, and C, with C having the worst prognosis.

ELISA	The enzyme-linked immunosorbent assay is a test that uses antibodies and color change to identify certain substances or cytokines.
Liver cirrhosis	The last stage of liver fibrosis that is accompanied by a reduced liver function is called liver cirrhosis.
Liver fibrosis	This is the formation of excess fibrous connective tissue (scar tissue) in the liver in a reparative or reactive process.
Liver steatosis	This is the abnormal accumulation of lipids in liver tissue, which is also called a fatty liver.
NAFLD	Nonalcoholic fatty liver disease is the term that encompasses simple steatosis, nonalcoholic steatohepatitis, and cirrhosis (as a result of steatohepatitis).
PBMC	Peripheral blood mononuclear cells are blood cells that have a round nucleus such as lymphocytes, monocytes, and macrophages.
Portal hypertension	Portal hypertension is hypertension (high blood pressure) in the portal venous system compared to the hepatic venous or inferior vena cava pressure, usually defined as an elevation of the hepatic venous pressure gradient (difference between wedged and free hepatic venous pressure) higher than 5 mmHg in patients with cirrhosis.
Visfatin	Visfatin is an adipokine (although this definition is under debate) and also goes by the name pre-B-cell colony-enhancing factor or nicotinamide phosphoribosyltransferase (Nampt). This protein is synthesized by adipose tissue, but also by many other tissues including PBMC, cartilage, and synovium. It was initially discovered as a molecule secreted by activated lymphocytes in bone marrow and is able to stimulate the formation of pre-B cells, but can also act as a proinflammatory cytokine able to induce IL-1β, IL-6, and TNFα.

Introduction

The liver has a unique vascular anatomy involving portal vein, hepatic artery, and hepatic veins. It is strategically located between the digestive system and the systemic circulation and has a unique metabolic activity in terms of the processes of degradation and synthesis. This activity does not only encompass hormones, metabolites, nutrients, and toxins but also cytokines. Levels of cytokines in peripheral blood are frequently used as surrogate markers to assess processes located in the liver. Considering that the liver can produce and clear cytokines, this assumption can, however, be questioned, and cytokines measured in peripheral blood might not always represent the cytokine profile present in hepatic or portal venous blood or in

liver tissue. Data on this issue are scarce. This chapter provides an overview of the available studies focusing on the cytokine differences, both in healthy livers and liver diseases, between the different body compartments: liver tissue, arterial blood, portal venous blood, hepatic venous blood, and peripheral blood (Table 1).

In a normal healthy liver, approximately 70% of incoming blood flows through the portal vein, collecting blood from the splenic and superior mesenteric vein, into the liver. The hepatic artery, which is one of three branches of the celiac trunk, delivers the remaining 30% of blood. After passing through the hepatic sinusoids, the blood is collected through three hepatic veins into the inferior vena cava. When the inferior vena cava reaches the heart, it does not only contain blood supplied from the hepatic veins, but is mixed with blood from the common iliac, gonadal, renal, suprarenal,

Table 1 Overview of present literature researching cytokine profile in the different compartments. Name of the first author, year of publication, and cytokines studied in the different compartments: portal venous (*PVS*), hepatic venous (*HVS*), systemic venous (*SVS*), arterial (*Art*), and intrahepatic (*IH*)

Name	Year	Cytokine	PVS	HVS	SVS	Art	IH
Douzinas	1997	IL-1β, IL-6, TNFα		X	X		
Narumi	1997	CXC10			X		X
Castelruiz	1999	IFNα, IFNβ			X		X
Nishioji	2001	CXC10			X		X
Poon	2003	$VEGF_{165}$			X		X
Wald	2004	CXC12			X		X
Soresi	2005	IL-6			X		X
Fontana	2007	IL-6, TNFα, MCP1, resistin, leptin	X			X	
Iavarone	2007	$VEGF_{165}$		X	X		X
Wieckowska	2008	IL-6			X		X
Sookoian	2009	ICAM1, CD40, PAI1			X		X
Wiest	2010	Visfatin, leptin, resistin, adiponectin	X	X	X	X	
Weigert	2010	Galectin-3	X	X	X		
Wiest	2011	IL-6	X	X	X		
Vuppalanchi	2011	Malondialdehyde		X	X		X
Wanninger	2011	Galectin-3	X	X	X		
Coulon	2012	TNFα, IL-6, VEGF, VGFR1, VGFR2			X		X
Karbaschian	2013	Visfatin	X		X		
Qian	2013	IL-17, IL-23			X		X
Vonghia	2015	IL-1β, IFNγ, TNFα, IL-4, IL-6, IL-10, IL-17a, IL-21, IL-23		X	X		
Verrijken	2014	PAI1			X		X
Porowski	2015	IL-6, TNFα, HGF, TGFβ	X	X	X	X	
Berres	2015	CXCL9	X	X	X		
Berres	2015	CXCL11	X	X	X		

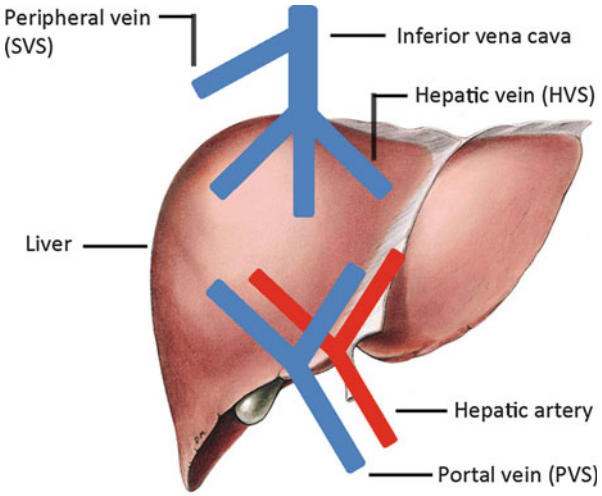

Fig. 1 Different cytokine compartments: portal venous system (*PVS*), hepatic venous system (*HVS*), peripheral venous system (*PVS*, e.g., antecubital vein), arterial blood, and hepatic tissue

inferior phrenic, and lumbar veins. Considering the different compositions of blood and the different origins of the blood, one can discern different compartments: portal venous blood/system (PVS), hepatic venous blood/system (HVS), peripheral or systemic venous blood/system (SVS), arterial blood, and hepatic tissue (Fig. 1).

Not only does the liver play an important role in several bodily functions from protein production and blood clotting to cholesterol, glucose, and iron metabolism but the liver also acts as an immunological organ, giving rise to many processes implicated in inflammation and immune control (Racanelli and Rehermann 2006). Nevertheless, the liver is not only an important site of synthesis but also the major clearance organ for several cytokines (Andus et al. 1991).

Differential levels of cytokines in different blood compartments may suggest the involvement of certain tissues in the local production of circulating cytokines. In a study of sepsis and colonic involvement, for example, it has been shown that following abdominal aortic surgery, levels of tumor necrosis factor (TNF) were higher in the portal vein than in systemic blood. The authors of this study hypothesize that the gut, suffering from hypoperfusion during sepsis, may be a source of cytokines that are subsequently removed from the blood by the liver (Cabié et al. 1993). Another study, on the other hand, showed no difference of TNFα, interleukin-1β (IL-1β), and IL-6 levels between portal blood and systemic blood in a murine model of sepsis through cecal ligation and puncture (Koo et al. 1999). Extrapolating these studies to the liver, it could be possible that in the condition of liver disease, the cytokine levels in the hepatic venous blood are higher compared to the portal blood and peripheral blood. Douzinas et al. supported this by demonstrating that in multiple-organ failure patients with hepatic involvement, IL-1β, IL-6, and TNFα levels were higher in hepatic venous blood compared to peripheral venous blood (Douzinas et al. 1997). In the next part of this chapter, we will solely focus on studies concerning primary liver diseases.

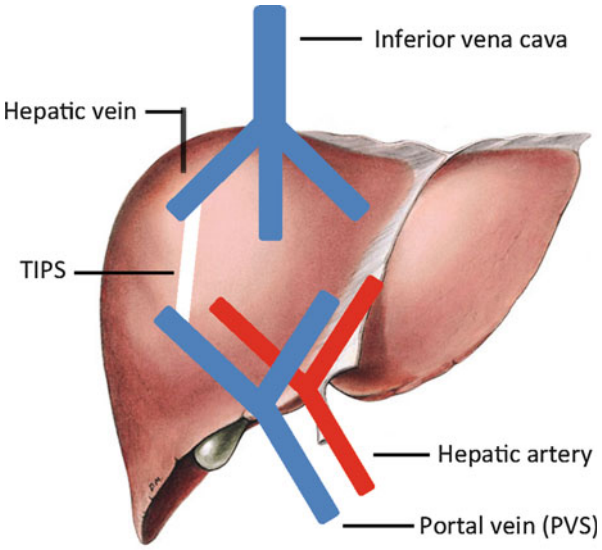

Fig. 2 Transjugular intrahepatic portosystemic shunt (TIPS). An artificial channel is created within the liver using balloon angioplasty and stent-graft technology that establishes a communication between the inflow portal vein and the outflow hepatic vein

Studies on this topic, however, are limited because of the difficulties in obtaining portal and hepatic venous blood, since surgery and transjugular endovascular catheterization are the only available methods. During upper abdominal surgery or liver surgery, portal and hepatic venous blood can be obtained. During transjugular intrahepatic portosystemic shunt (TIPS) placement, a bypass is made between a portal vein branch and a hepatic vein (Fig. 2). During TIPS implantation, samples of one of the hepatic veins not drained by the TIPS and of the portal vein can be made. During transjugular liver biopsy, only hepatic venous blood and not portal venous blood can be obtained.

Evaluation of Portal and Systemic Venous Blood Compartments

Karbaschian et al. studied the difference of the adipokine visfatin in portal and peripheral blood in an obese population (Karbaschian et al. 2013). Samples were obtained simultaneously from an antecubital vein and the portal vein in a population of severely obese patients during restrictive bariatric surgery. They observed significantly higher visfatin concentrations in the portal vein, compared to the peripheral veins. The authors postulate that the higher levels of visfatin in the portal vein provide evidence that visceral adipose tissue is the major secretory source of visfatin in humans. Remarkably, there was no significant correlation between portal and systemic levels of visfatin. The study additionally tried to assess a possible relationship of visfatin with systemic inflammation and insulin resistance. They discovered that, while systemic levels of visfatin were significantly correlated with circulating levels of C-reactive protein (CRP), there were no significant correlations between portal levels of visfatin with systemic levels of CRP concentrations. No associations

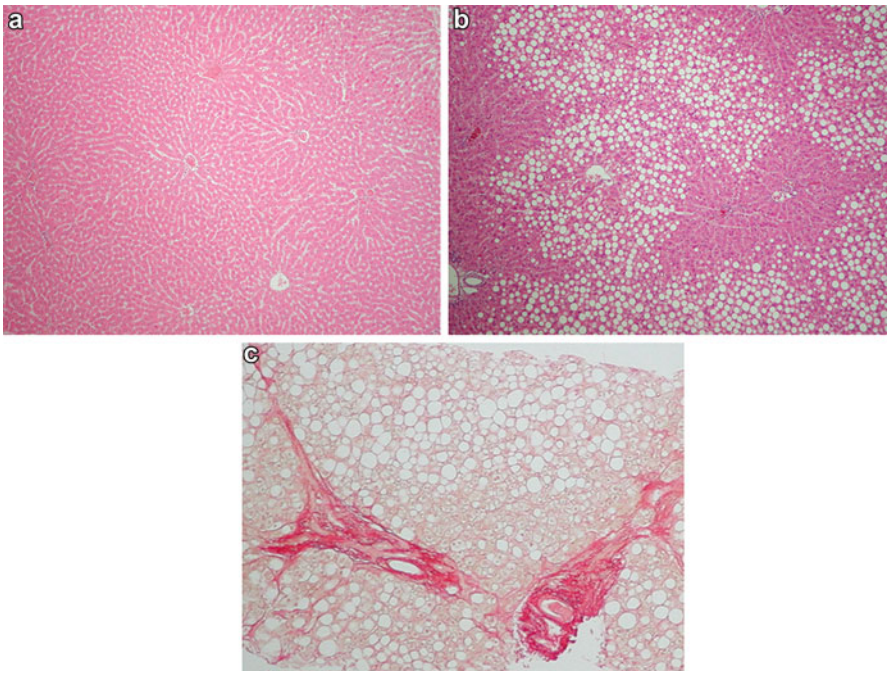

Fig. 3 Microscopic images of normal liver, fatty liver, and liver fibrosis. (**a**) Normal liver tissue without fat droplets, inflammation, or fibrosis (H&E staining). (**b**) Simple liver steatosis without overt signs of inflammation/NASH (H&E staining). (**c**) Liver fibrosis with periportal fibrosis and portal-portal septa (Fouchet staining)

between visfatin and parameters of the metabolic syndrome were detectable. Unfortunately, the authors did not describe/investigate liver function, the presence of nonalcoholic fatty liver disease (NAFLD), or fibrosis in their patient population (Fig. 3). This study clearly demonstrates that a good correlation between portal and systemic venous blood cytokine levels is not always present, illustrating the role of the liver in extraction and degradation as well as production of cytokines.

Evaluation of Different Venous Blood Compartments

Interleukin-6 is a pleiotropic cytokine and is produced by different cells like endothelial cells, adipocytes, and cells of the immune system. An important function of IL-6 is the induction of acute phase response proteins like fibrinogen and CRP in the liver. In healthy individuals the splanchnic organs and the kidney are responsible for diluting systemic IL-6 levels. Wiest et al. investigated the concentrations of IL-6 in portal, hepatic, and systemic venous serum of patients with liver cirrhosis and patients with a normal liver function (Wiest et al. 2011). In cirrhotic patients, PVS-IL-6 was significantly higher than HVS-IL-6 and SVS-IL-6, whereas

HVS-IL-6 was lower than SVS-IL-6. Based on the different concentrations, the authors calculated that the liver in the cirrhotic population removed around 6.3% of PVS derived IL-6. In the healthy liver population, about 43% of IL-6 was removed indicating significantly impaired hepatic IL-6 clearance in patients with liver cirrhosis. Subgroup analysis showed that HVS-IL-6 was almost 25% lower than in PVS in patients with Child-Pugh score A compared to patients with Child-Pugh score C where IL-6 was similarly abundant in the two blood compartments. Hepatic venous pressure gradient did not correlate with the degree of hepatic IL-6 removal excluding hepatic shunting as the principal cause of impaired IL-6 uptake. A limitation of this study is that only a small number of liver-healthy donors were included, who furthermore suffered from malignant tumors, which might influence the cytokine profile. This study demonstrated an impaired cytokine clearance in cirrhotic patients, which worsens with increasing Child-Pugh score.

Wiest et al. investigated the serum concentrations of the adipokines visfatin, leptin, resistin, and adiponectin in patients with liver cirrhosis undergoing TIPS implantation (Wiest et al. 2010). They also collected blood (SVS, HVS, and PVS) in a "healthy normal-weight" cohort of patients who were submitted to liver surgery for nonhepatic diseases and who had a normal liver function. In the patients with a normal liver function, there was no significant difference between adipokine concentrations in the different compartments. In patients with liver cirrhosis, resistin levels and adiponectin levels were similarly almost identical in all blood compartments. Visfatin levels, on the other hand, were significantly higher in portal and hepatic venous blood of cirrhotic patients when compared with systemic blood. This might indicate that visfatin is secreted in higher quantities by the visceral adipose tissue than by peripheral subcutaneous adipose tissue. Visfatin levels were not different in portal blood compared to hepatic venous blood in patients with liver cirrhosis, indicating that there is no hepatic clearance of this adipokine. Patients with a normal liver function had significantly higher overall concentrations of visfatin when compared with patients with liver cirrhosis, indicating that visfatin levels are anyhow severely decreased in liver cirrhosis. The mechanism of this is unclear, as the liver is not a known source of visfatin production. Subgroup analysis, on the other hand, showed a higher visfatin level in patients with Child-Pugh score C, compared to patients with Child-Pugh score A, contrarily to what would be expected. Leptin levels were significantly higher in SVS when compared with PVS and HVS. This is in line with leptin secretion dynamics, given that leptin is secreted from peripheral subcutaneous adipose tissue stores in higher quantities than from visceral adipose tissue (Van Harmelen et al. 1998). Leptin does not seem to undergo any hepatic clearance because HVS and PVS levels are almost identical. Moreover, there was no difference of leptin concentrations between cirrhotic patients and patients with a normal liver function, nor was there one for adiponectin. Resistin levels, on the other hand, were significantly higher in the TIPS patients compared to the control group, indicating that resistin levels are increased in liver cirrhosis. This is congruent with the subgroup analysis, showing higher levels of resistin in patients with Child-Pugh C, compared to patients with Child-Pugh A. None of the adipokines were significantly correlated with the portocaval pressure gradient. This study

demonstrates that not only the location of blood sampling but also the presence of cirrhosis, and the extent of the liver disease will influence the blood level of certain adipokines. Liver function, reflected by the Child-Pugh score, might have a congruent result with the presence of cirrhosis, like resistin, or an incongruent effect like in case of visfatin.

Galectin-3 is a member of the β-galactosidase-binding lectin family and controls crucial cellular functions including cell adhesion, survival/apoptosis, regulation of adaptive immunity, and macrophage activation. Galectin-3 also has a role in the activation and transdifferentiation of hepatic stellate cells to myofibroblasts and in the progression of liver fibrosis (Serizawa et al. 2015). Previous studies have shown that galectin-3 is found highly abundant in the colon and visceral fat suggesting that these tissues may release galectin-3 into the portal vein. Blood monocytes also synthesize galectin-3 and may contribute to systemic levels. Weigert et al. investigated galectin-3 in different blood compartments and found in patients undergoing liver surgery for metastases of extrahepatic malignant tumors the lowest galectin-3 level in HVS, a significantly higher level in SVS, and the highest in PVS (Weigert et al. 2010). Similar results were obtained in the cirrhotic patients with the lowest galectin-3 level in HVS, significantly higher levels in SVS, and highest in PVS (significant compared with HVS, not significant compared with SVS). In this study, the proportion of galectin-3 concentrations did not differ between the different blood compartments between cirrhotic patients and patients with a healthy liver.

Evaluation of Arterial and Venous Blood Compartments

To study the role of visceral fat in the production of adipokines, the concentration of the adipokine in arterial blood and in portal blood can be compared. Fontana et al. evaluated the relative contribution of inflammatory adipokines [IL-6, TNFα, macrophage chemoattractant protein-1 (MCP-1), resistin, and leptin] from visceral fat in insulin-resistant, extremely obese subjects who underwent open gastric bypass surgery (Fontana et al. 2007). They found significantly higher plasma concentrations of IL-6 in the portal vein compared to peripheral artery blood in obese subjects, demonstrating that visceral fat is an important source of IL-6 production in obese people. Portal vein IL-6 concentrations also correlated directly with systemic CRP concentrations in obese subjects, whereas arterial IL-6 concentrations did not. This suggests a potential mechanistic link between visceral fat mass and systemic inflammation in human subjects. Plasma leptin concentrations were lower in the portal vein than in peripheral artery blood. The authors explain this by the observation that the expression of the *ob* gene, which produces leptin, and leptin secretion are lower in omental than subcutaneous fat (Ramis et al. 2005; Van Harmelen et al. 1998). In contrast to IL-6 and leptin, the plasma concentrations of other adipokines, such as TNFα, MCP-1, and resistin, were similar in the portal vein and the peripheral artery. This study shows us that systemic levels of certain cytokines, in this case IL-6, do not necessarily correlate well with systemic inflammation and that determination of a cytokine level distal from the organ of interest could be more representative.

To develop a model of cytokine elimination by the liver, Porowski et al. collected blood from the portal, hepatic, and peripheral vein and the hepatic artery to determine the concentrations of IL-6, TNFα, hepatocyte growth factor (HGF), and transforming growth factor-beta (TGFβ) (Porowski et al. 2015). In donors, significantly lower levels of IL-6, TNFα, HGF, and TGFβ were detected in the hepatic vein compared to the portal vein. In patients with cirrhosis, there were no significant differences of IL-6, TNFα, and TGFβ levels between portal and hepatic veins. Significantly higher levels of HGF in hepatic compared to portal vein were observed. The authors developed a mathematic model of cytokine elimination incorporating a model of in-hepatic synthesis and degradation, as well as cytokine levels of portal vein, hepatic vein, and hepatic artery. In healthy livers, elimination of the cytokines prevailed over their synthesis, as reflected by the positive values of elimination ratios. In the cirrhotic liver, elimination ratios of IL-6, HGF, and TGFβ were negative, indicating the prevalence of intrahepatic synthesis of cytokines over their removal. The elimination ratio of TNFα, on the other hand, was positive in the cirrhotic population. This study shows us that measuring cytokines in several blood compartments can give insight in their production and elimination process, both in normal and pathological (e.g., cirrhosis) conditions.

Evaluation of Hepatic Tissue and Blood Compartments

Circulating cytokines can represent the tip of the iceberg (Cavaillon et al. 1992). Once cytokines are produced, they can be detected within the producing cells and/or on the cell surface for some cytokines, such as IL-1, TNFα, IL-10, IL-15, or interferon gamma (IFNγ), which can exist as membrane forms. Once cytokines are released, they are present within a cellular environment and can be efficiently trapped by surrounding cells that possess specific receptors. Detection of cell-associated cytokines hence should not only be interpreted as an indication of the cellular source of a given cytokine because internalization of receptor-bound environmental cytokines can also occur. Circulating cytokines are also not always in line with the hepatic cytokine profile. Malondialdehyde (MDA), not a cytokine, but an end product of lipid peroxidation, has been used as a biomarker for oxidative stress. Questioning the validity of using peripheral MDA levels as markers of oxidative stress as a reflection of hepatic oxidative stress, Vuppalanchi et al. sampled peripheral and hepatic venous blood, as well as liver tissue during transjugular pressure measurements in patients with various liver diseases (Vuppalanchi et al. 2013). They observed that peripheral venous MDA levels exhibited a statistically significant correlation with hepatic venous MDA content. There was, however, no relationship between peripheral venous and hepatic tissue MDA content, nor was there a significant relationship between hepatic venous and hepatic tissue MDA content. They only found a relationship between hepatic tissue MDA content and hepatic venous MDA levels, after subgroup analysis, in patients without portal hypertension. They conclude that peripheral venous measurements of oxidative stress are not a valid reflection of hepatic tissue oxidative stress and might only be suitable for

assessing hepatic tissue oxidative stress in patients without portal hypertension. In this study, the hepatic venous blood, which represents the outflow tract of the liver, was not representative of the liver tissue compartment profile. Portal hypertension and possible redirection of blood flow might have an influence on blood levels of cytokines or other substances.

Wanninger et al. evaluated galectin-3 levels in peripheral, portal, and hepatic blood during TIPS placement in patients with liver cirrhosis (Wanninger et al. 2011). Patients undergoing liver surgery for metastases of extrahepatic malignant tumors were used as a control group. In the healthy group, galectin-3 was higher in PVS than in HVS and SVS, suggesting that the liver removes galectin-3. In the cirrhosis group, galectin-3 was similar in SVS to HVS and PVS concentrations and was similar to even higher in HVS compared to PVS. HVS and SVS galectin-3 levels were significantly higher in patients with liver cirrhosis than controls, whereas PVS levels were not altered. These results indicate that galectin-3 is not extracted by the cirrhotic liver, but even seems to be produced in the liver of some patients with liver cirrhosis. Immunohistochemistry analysis confirmed that galectin-3 is not expressed in hepatocytes of a healthy liver, but is induced in hepatocytes of cirrhotic livers, which confirms the findings of previous studies (Hsu et al. 1999). This study shows that if one would determine galectin-3 levels in peripheral blood or hepatic venous blood, they would find higher levels in the cirrhotic population compared to the healthy population, which would be a congruent result with elevated galectin-3 in the liver tissue. But if one would investigate galectin-3 levels in portal blood, they would find no difference between the two populations.

Qian et al. investigated the expression of IL-23 and IL-17 in the peripheral blood of patients with primary biliary cirrhosis (PBC), an autoimmune disease of the liver that is characterized by a T-lymphocyte-mediated attack on small intralobular bile ducts (Qian et al. 2013). They evaluated messenger ribonucleic acid (mRNA) expressions in peripheral blood mononuclear cells (PBMC), as well as serum levels through enzyme-linked immunosorbent assay (ELISA) and immunohistochemical staining of liver tissue. mRNA expression levels of IL-23 and IL-17 in PBMCs from PBC patients were significantly increased, as were the PBC patients' serum levels of IL-23 and IL-17, compared to those in the healthy group. When performing analysis on the different stages of PBC, they found lower values of IL-17 and IL-23 in patients with advanced disease, compared to patients with early disease. Immunohistochemical staining, however, showed significantly more IL-23+ and IL-17+ mononuclear cells in portal areas in liver tissues in advanced stages of the disease compared to the early stages. The authors suggest that this might be attributed to the migration of T cells during the progression of disease. This study shows us that elevation of cytokine levels in patients with a certain liver disease does not necessarily correlate with the histological severity of the disease.

Chemokines, a family of structurally related proteins, are considered as master regulators of immune cell trafficking in the body under both physiological and pathological conditions. Most chemokines have four characteristic cysteines, and, depending on the motif displayed by the first two cysteines, they have been classified into CXC, CC, XC, and CX3C chemokines. Chemokines are then named in a

numerical manner, such as CXCL 1-16. Nishioji et al. studied the serum level of CXCL10 (also called interferon-inducible protein-10) and its expression in the liver in patients with autoimmune hepatitis (AIH) and PBC (Nishioji et al. 2001). The serum levels of CXCL10 were significantly elevated in AIH and PBC patients compared to healthy controls. The serum level also increased in proportion to the degree of histological activity and was significantly correlated with the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In situ hybridization of CXCL10 mRNA similarly demonstrated expression in the hepatocytes around intralobular focal necrosis or lobular necrosis. These results are in line with another study investigating CXCL10 in patients with chronic hepatitis B (CHB) and C (CHC) where CXCL10 mRNA was also expressed in the liver of CHB and CHC patients and where the serum level of CXCL10 was elevated in chronic viral hepatitis patients. Similarly, CXCL10 serum levels related positively to serum AST and ALT levels and the degree of necroinflammatory activity (Narumi et al. 1997). Wald et al. studied the role of CXCL12 in chronic viral hepatitis (Wald et al. 2004, 2007). CXCL12 is constitutively produced in healthy individuals in the bone marrow, lung, and liver. In normal healthy liver tissue sections, the expression of CXCL12 is restricted to bile duct epithelial cells in the portal tracts. In mildly to moderately inflamed liver samples (based on the Knodell scoring system), few inflammatory foci were detected, and a beginning of bile duct proliferation was observed. In this phase of the disease, CXCL12 was mainly observed in the inflammatory foci of proliferating bile ducts, and there was some staining of some blood vessels. In highly inflamed and cirrhotic liver samples, an anatomical redistribution of CXCL12 expression was evident with proliferating bile ducts and blood vessels along the fibrotic septi strongly expressing CXCL12. In accordance with the results obtained by immunohistochemistry, they found a significant elevation in CXCL12 levels in the plasma of CHC patients with significant and advanced fibrosis relative to healthy individuals and relative to patients with mild fibrosis. Similar results were observed when assessing plasma levels of CXCL12 in hepatitis B-infected individuals with advanced fibrosis compared with CHB patients with no or mild liver fibrosis. These results also support the association between CXCL12 expression and progression of liver fibrosis, while the elevated levels of CXCL12 in the plasma of hepatitis C-infected individuals are detected regardless of fibrosis severity. Based on the congruent results of elevated CXCL10 and CXCL12 in serum and in liver tissue and their association with liver disease severity, the authors suggest that in this case a combinatorial assessment may be indicative of disease activity and fibrosis development. These studies showed a congruent result between serum cytokine level and intrahepatic cytokines, as well as a correlation with disease severity and fibrosis.

Coulon et al. investigated the role of inflammatory (TNFα, IL-6) and angiogenic [vascular endothelial growth factor (VEGF) A] cytokines and soluble VEGF receptors 1 and 2 (sVEGFR1, sVEGFR2) in the serum of an obese population with simple steatosis and nonalcoholic steatohepatitis (NASH) compared to healthy controls (Coulon et al. 2012) (Fig. 4). The study also included the detection of TNFα, VEGF, VEGFR1, and VEGFR2 gene expression in the liver of the patients with

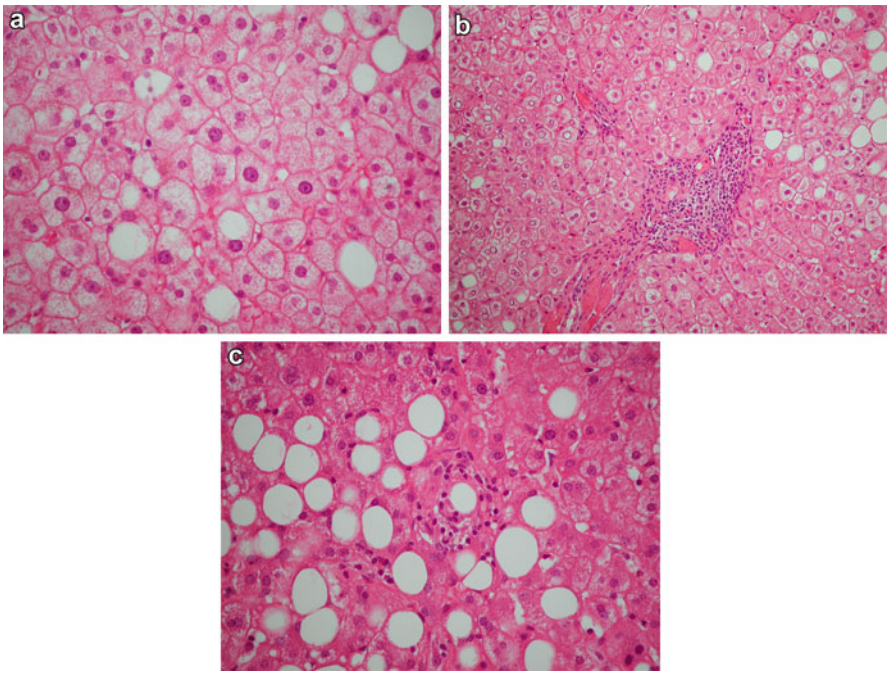

Fig. 4 Microscopic images of liver tissue with signs of nonalcoholic steatohepatitis (H&E staining). (**a**) Ballooning of hepatocytes: rounded hepatocytes with pale cytoplasm and normal or enlarged size. (**b**) Portal inflammation with inflammatory cells around a portal branch. (**c**) Lobular inflammation with inflammatory cells within the liver lobule

simple steatosis and NASH, but not in healthy livers. Serum TNFα levels were the highest in the NASH group and were significantly higher compared to the control group. TNFα concentrations were increased in the serum of simple steatosis patients compared to the control group, but this difference did not reach statistical significance. There was no significant difference in serum TNFα concentrations between simple steatosis and the NASH group. On the other hand, liver TNFα mRNA expression was significantly higher in NASH patients compared to simple steatosis patients, which is hence not in line with the data of the serum levels. Both simple steatosis patients and NASH patients had increased VEGF serum levels compared to the control group, with no significant difference between the simple steatosis and the NASH group. Similarly, sVEGFR1 serum levels were increased in both simple steatosis patients and NASH patients compared to the control group, with no significant difference between simple steatosis and the NASH group. No significant differences were detected between groups regarding VEGFR2 serum levels. VEGF and VEGFR1 mRNA expression were significantly higher in simple steatosis patients compared to NASH patients, whereas VEGFR2 expression was only slightly increased in the simple steatosis patients compared to the NASH patients.

Although no significant differences are present between the simple steatosis group and the NASH group in VEGF, sVEGFR1, and sVEGFR2 in serum, mRNA expression of these cytokines was higher in the simple steatosis group, opposite to the situation of TNFα. IL-6 serum levels were significantly higher in both the NASH group and simple steatosis group compared to controls with no significant difference between the simple steatosis and the NASH group. The expression of IL-6 was below the detection limit, so no correlation could be made with intrahepatic IL-6 expression in this population. This study demonstrates that the serum levels of certain cytokines can be different from the intrahepatic expression. It also shows that the cytokine level differences between NASH and simple steatosis depend on the type of cytokine.

Soresi et al. studied IL-6 and its receptor (IL-6R) in patients with cirrhosis and hepatocellular carcinoma (HCC), cirrhosis without hepatocellular carcinoma (LC) and controls (Soresi et al. 2006). Immunohistochemistry analysis showed a medium to strong cytoplasmic and membrane reactivity for IL-6 and IL-6R respectively, in at least 40% of cases of HCC tissue, where liver cirrhosis patients and controls were negative for IL-6 or showed a very mild and focal dot-like cytoplasmic reaction for IL-6R. Peripheral blood serum IL-6 levels in the HCC group were significantly higher than those in LC and control groups, and serum levels were significantly higher in LC compared to the control group. A significant difference was found in sIL-6R serum levels between the HCC group and controls, but not between the HCC and LC groups. When the patients with HCC were divided into groups according to Okuda's classification (a prognostic score that incorporates tumor features and the degree of underlying cirrhosis), a significant serum increase of IL-6 and sIL-6R was observed from stage I to stage III. When dividing the patients into groups according to the Child-Pugh classification, the median sIL-6R value decreased from class A to C in the cirrhotic group, but increased in the HCC group. This study shows a congruent overrepresentation of IL-6 in serum, as well as in liver tissue in patients with liver cirrhosis and hepatocellular carcinoma, suggesting an increased production of this cytokine by neoplastic cells. These results also indicate that in cirrhotic patients sIL-6R production and release decrease as the disease progresses and the liver parenchymal mass is reduced. On the other hand, in HCC patients, increased sIL-6R serum concentrations might be due to the increasing tumor mass. In this study, serum cytokine presentation was dependent on reduced liver function and other disease-related alterations (e.g., the presence of HCC) that can have opposite effects complicating the interpretation of serum levels and hence their validity as markers of disease severity.

Wieckowska et al. evaluated the occurrence of IL-6 expression in the liver (immunohistochemistry) and peripheral blood (ELISA) in human NAFLD and compared patients with NASH, simple steatosis, and healthy livers (Wieckowska et al. 2008). They found a significant increase of IL-6 expression in the livers of patients with NASH compared to patients with simple steatosis or normal biopsies. A positive correlation was observed between hepatocyte IL-6 expression and the degree of inflammation (NAFLD Activity Score) and the stage of fibrosis. Liver IL-6 expression also correlated positively with plasma IL-6 levels. And plasma IL-6

levels correlated positively with the stage of fibrosis, after multiple regression analysis. In patients with type II diabetes mellitus (T2D), both hepatic and circulating IL-6 expression were significantly higher compared with those without evidence of T2D. This study showed that hepatic IL-6 correlates well with the presence and severity of NASH, but although hepatic and plasma IL-6 correlate well, plasma IL-6 does not correlate significantly after multiple regression analysis with the presence and severity of NASH. This study also showed a congruent elevation of hepatic and serum IL-6 in patients with T2D.

Sookoian et al. explored circulating levels of biomarkers of atherosclerosis (soluble intercellular adhesion molecule, sICAM-1; plasminogen activator inhibitor, PAI-1; and soluble CD40 ligand, sCD40L) through ELISA on serum samples, as well as the liver protein expression of ICAM-1, CD40, and PAI-1 through immunostaining in patients with different histological forms of NAFLD and in control livers (Sookoian et al. 2010). sICAM was significantly higher in NAFLD patients compared to controls. Within the NAFLD group, sICAM was higher in the NASH group compared to the simple steatosis group. A positive correlation was observed between sICAM-1 and the degree of liver steatosis and between sICAM-1 and the severity of necroinflammatory activity. There was no association between sICAM-1 and the degree of hepatic fibrosis. sCD40L and circulating PAI-1 levels were both significantly higher in NAFLD patients compared to healthy individuals, but there was no association with the degree of necroinflammatory activity or with the fibrosis stage. Immunohistochemistry was separately assessed in hepatocytes and in the lobular inflammatory infiltrate. ICAM-1 and hepatocyte PAI-1 expression were higher in NAFLD compared to control patients, and ICAM-1 was significantly higher in NASH compared to simple steatosis. There was no significant difference between controls and NAFLD patients in PAI-1 staining in lobular inflammatory infiltrate and in CD40 staining. No association was found between the degree of liver steatosis and ICAM-1 in hepatocytes, but there was a correlation between the level of liver ICAM-1 expression in the lobular inflammatory infiltrate and the degree of liver steatosis. Liver ICAM-1 expression also positively correlated with the severity of necroinflammatory activity, but not with fibrosis. Intriguingly, no significant correlation was observed between the level of sICAM-1, PAI-1, and sCD40L in serum and the degree of liver protein expression. Nevertheless, a positive correlation was found between circulating levels of sCD40L and both liver expression of ICAM-1 and PAI-1. Another study also investigated PAI-1 in serum and liver tissue of obese patients without steatosis, patients with simple steatosis, and patients with NASH (Verrijken et al. 2014). The authors did find significantly increased levels of systemic PAI-1 with increasing severity of steatosis, lobular inflammation, ballooning, and fibrosis. Patients with NASH had significantly higher systemic PAI-1 values than those with a healthy liver. Liver PAI-1 gene expression was determined by RNA extraction and real-time quantitative PCR and was significantly higher in patients with NASH, compared to those with borderline or no NASH. The discrepancy between this study and the former studies might be explained by differences in methodology to assess liver protein expression [e.g., gene expression by reverse

transcription polymerase chain reaction (PCR) versus immunostaining]. The first study also shows an important effect of the histological distribution within the liver on the degree of correlation.

VEGF is a potent angiogenic factor with five molecular isoforms, generated by alternative splicing of VEGF mRNA. $VEGF_{165}$ is secreted into the circulation and is the predominant isoform secreted by most tumors. Iavarone et al. investigated the expression of $VEGF_{165}$ in plasma and liver tissue in patients with HCC, with cirrhosis, or with chronic hepatitis (Iavarone et al. 2007). $VEGF_{165}$ mRNA was overexpressed in the HCC tissues compared with the nonmalignant tissues (cirrhotic and chronic hepatitis grouped) and also higher in the HCC than in the cirrhotic tissues. $VEGF_{165}$ was significantly higher in the cirrhotic tissues compared with the chronic hepatitis tissues. $VEGF_{165}$ was also higher in the cirrhotic tissues surrounding HCC than in the HCC-free cirrhotic tissues. The plasma levels of $VEGF_{165}$ in hepatic and femoral veins were higher in patients with HCC than in cirrhosis. Femoral levels of $VEGF_{165}$ significantly correlated with hepatic vein levels in both HCC and cirrhosis. $VEGF_{165}$ was significantly lower in the femoral veins compared to the hepatic veins. This study could not find a direct correlation between tumor expression and serum levels of VEGF, contradictory to a study of Poon et al. who did find a correlation between serum levels and mRNA expression in tumor tissue (Poon et al. 2003). The data of Iavarone et al. strongly argue in favor of circulating $VEGF_{165}$ to reflect increased expression of VEGF by neoplastic liver cells. Cirrhotic patients showed an inverse correlation between hepatic vein levels of $VEGF_{165}$ and hepatic venous pressure gradient (HVPG) scores, while no such correlation was found in HCC patients. This suggests that in these patients plasma levels of $VEGF_{165}$ were mainly influenced by $VEGF_{165}$ synthesis by neoplastic liver cells rather than by portal hypertension. This study shows that the presence of portal hypertension with collateral circulation might alter the cytokine distribution and their correlation between the different compartments in specific patient groups.

Vonghia et al. investigated the cytokine profile in peripheral and hepatic venous blood of NAFLD/NASH patients undergoing transjugular liver biopsy and compared them with histology and hemodynamic and metabolic parameters (Vonghia et al. 2014). They determined nine cytokines (fluorescent bead on serum: IL-1β, IFNγ, TNFα, IL-4, IL-6, IL-10, IL-17a, IL-21, and IL-23) and studied the balance between pro- and anti-inflammatory systems through the IFNγ/IL-4 ratio (indicative of the Th1/Th2 balance), the (TNFα+IL-6+IL-23)/IL-10 ratio (indicative of the M1/M2 balance), and the IL-10/IL-17 ratio. When considering the whole group of patients, no difference between cytokine measurements in peripheral blood and hepatic venous blood reached statistical significance, except for IL-6. IL-6 was higher in SVS than in HVS. When considering the individual cytokines, patients with NASH displayed lower values of SVS-IL-21 in comparison with patients affected by simple steatosis. When looking at HVS/SVS ratios, diabetic patients showed lower values of IL-23 and higher values of IL-4 than nondiabetic patients. Patients with diabetes mellitus showed higher levels of HVS-IL-4 and HVS-IFNγ compared to nondiabetic patients. When considering the cytokine ratios, a significant lower SVS-IL-10/IL-17

ratio and higher SVS-M1/M2 ratio were observed in NASH versus simple steatosis. The HVS-M1/M2 ratio was also significantly lower in patients with portal hypertension in comparison with patients without portal hypertension. Diabetic patients showed an increase of the PVS-Th1/Th2 ratio in comparison with nondiabetics. When looking at correlations, both the HVS-M1/M2 ratio and the SVS-M1/M2 ratio correlated positively with fasting insulin, whereas only the PVS-M1/M2 ratio correlated with steatosis grade. Both SVS- and HVS-IL-10/IL-17 ratios correlated negatively with fasting insulin. This study shows that, although in the overall patient group there was no significant difference between peripheral and hepatic venous cytokine levels, some individual cytokines might differ between groups, giving information that is potentially relevant to understand disease pathophysiology. It also showed a specific correlation between intrahepatic cytokines and histological, hemodynamic, and metabolic characteristics of the disease.

IFNα and IFNβ, two members of the type I IFN family, are produced by a great diversity of cells in response to viral infections. While IFNβ is a glycoprotein product of a single gene, IFNα is a family of related polypeptides (subtypes), each encoded by a separate gene. Castelruiz et al. analyzed the IFNα subtypes and the mRNA levels of type I IFNs in samples of normal liver tissue and in livers from patients with chronic hepatitis C (Castelruiz et al. 1999). They performed similar studies in PBMC from patients and controls. They observed that 98 of the 100 clones from normal liver tissue corresponded to the IFNα5 subtype. However, in livers with chronic hepatitis C and in PBMC from controls and patients, a variety of subtypes, in addition to IFNα5, were detected, suggesting a participation of infiltrating leukocytes in the production of IFNα in livers with chronic hepatitis C. As compared with controls, patients with chronic hepatitis C showed a significant increase in IFNβ mRNA in both the liver and PBMC, while IFNα mRNA was significantly increased in PBMC but markedly reduced in liver tissue. Thus, HCV infection causes opposite changes of IFNα and IFNβ in hepatic tissue, decreasing the expression of the former while increasing the latter. This study showed that even within a family of cytokines, one (IFNβ) can have congruent levels between liver tissue and blood, while the other (IFNα) has incongruent levels in the same disease.

Apart from the difficulties of obtaining portal and hepatic venous blood, cytokine studies are also hindered by the different methods of cytokine detection, which must be taken into account when comparing studies encompassing cytokine research. mRNA expression may not necessarily indicate that the respective protein is really secreted from tissue into the downstream vein in higher amounts. Gene transcription could occur without protein translation. ELISA kits from different manufacturers can measure substantial differences in serum levels of cytokines, due to the use of different sets of antibodies with different affinities for the same antigen. Additionally, there is also the difference between cytokine levels studied in serum, with those studied in peripheral blood mononuclear cells. All these factors must be taken into account before drawing firm conclusions on associations or a fortiori on the role of cytokines in organ-specific pathophysiological processes.

Conclusion

Hepatic venous blood represents the outflow tract of the liver and hence theoretically allows assessing liver site-specific mechanisms more accurately. Similarly, when expecting the release of cytokines from tissue into the downstream vein, the portal vein should represent the situation of the visceral fat, intestines, spleen, and pancreas. The data reviewed in this chapter show that the relationship or correlation between portal venous blood, hepatic venous blood, systemic venous blood, and arterial blood is not always straightforward. Furthermore, there is not per se a relationship between cytokine levels in blood compartments and the intrahepatic cytokine profile. The studies reviewed demonstrate that this relationship can depend on the type of cytokine, presence of liver disease, disease severity, portal pressure, and/or liver function. By consequence, when studying the hepatic cytokine profile, one should be cautious to not simply extrapolate the cytokine presentation in blood to the situation in liver tissue.

Although practical difficulties and ethical limitations might hinder large studies, there is still a need for more extensive research, encompassing a wider variety of cytokines and different liver diseases. This type of studies give valuable insights in the differential role of the different body compartments, especially the liver, intestine, and adipose tissue, and help unraveling their complex interplay that cannot be assessed by a simple measurement in peripheral blood.

Potential Applications to Prognosis, Other Diseases, or Conditions

The knowledge of cytokines in the pathophysiology of diseases is expanding and is opening up new angles for diagnosis and therapy. More research is needed into the field of cytokines as markers of liver disease. The studies in this chapter show us that peripheral blood is not always a good indicator for the intrahepatic cytokine profile. The same might be true for cytokines as predictors of prognosis. When a cytokine determined in peripheral blood does not have a good predictive value, the same cytokine determined in portal of hepatic venous blood might have. One study investigated CXCL9, a CXCR3 ligand in blood obtained during TIPS placement in a cirrhotic population (Berres et al. 2015). They found that before TIPS placement, portal levels of CXCL9 revealed a predictive value for 6-month and 2-year survival in their cohort of patients with severe portal hypertension receiving TIPS, which was superior to the established MELD (model for end-stage liver disease) and Child-Pugh scoring system. Another study determined CXCL11, another CXCR3 ligand, in the same study setting (Berres et al. 2016). They found that hepatic CXCL11 levels before TIPS placement and low portal CXCL11 levels after TIPS predicted long-time survival.

Although the portal and hepatic venous levels of cirrhotic patients without TIPS could seem desirable, it is unpractical and unethical to obtain this in the usual clinical

setting. How future findings will impact portal venous or hepatic venous cytokine levels as biomarkers remains to be seen.

Sampling of blood in the different compartments can also be used to study the kinetics of cytokines. It can give valuable information of the differential role of the different body compartments, especially the liver, intestine, and adipose tissue, and shed light on their complex interplay that cannot be assessed by a simple measurement in peripheral blood.

Summary Points

- This chapter focuses on the differences of cytokine presentation between portal venous, hepatic venous, systemic venous and arterial blood, and hepatic tissue.
- Only invasive methods such as abdominal surgery, transjugular hepatic venous catheterization, or TIPS placement allow sampling of portal or hepatic venous blood.
- Theoretically, portal blood is a better representation of the situation in the gastrointestinal tract and the spleen than peripheral blood.
- Theoretically, hepatic venous blood is a better representation of the situation in the liver than peripheral blood.
- The blood level of certain cytokines can be influenced by the presence of liver disease, severity of liver disease, liver function, and portal pressure.
- There is not always a good correlation between cytokines in the different blood compartments.
- There is not always a good correlation between cytokines in the blood compartments and hepatic tissue.
- Correlation between cytokine levels of blood compartments and liver can change depending on the presence and severity of liver disease, liver function, and portal hypertension.
- Differences in cytokine levels in several blood and tissue compartments can give valuable insights in their respective sites of production and degradation and help unraveling their complex regulation in both physiological and pathological conditions.

References

Andus T, Bauer J, Gerok W. Effects of cytokines on the liver. Hepatology. 1991;13(2):364–75.

Berres ML, Lehmann J, et al. CXCL11 levels predict survival in cirrhotic patients with TIPS. Liver Int. 2016;36(3):386–94.

Berres M-L, Asmacher S, et al. CXCL9 is a prognostic marker in patients with liver cirrhosis receiving transjugular intrahepatic portosystemic shunt. J Hepatol. 2015;62(2):332–9.

Cabié A, et al. High levels of portal TNF-alpha during abdominal aortic surgery in man. Cytokine. 1993;5(5):448–53.

Castelruiz Y, et al. Interferon alfa subtypes and levels of type I interferons in the liver and peripheral mononuclear cells in patients with chronic hepatitis C and controls. Hepatology. 1999; 29(6):1900–4.
Cavaillon J, et al. Circulating cytokines: the tip of the iceberg? Circ Shock. 1992;38(2):145–52.
Coulon S, et al. Evaluation of inflammatory and angiogenic factors in patients with non-alcoholic fatty liver disease. Cytokine. 2012;59(2):442–9.
Douzinas E, et al. The regional production of cytokines and lactate in sepsis-related multiple organ failure. Am J Respir Crit Care Med. 1997;155:53–9.
Fontana L, et al. Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. Diabetes. 2007;56:1010–3.
Hsu DK, et al. Galectin-3 expression is induced in cirrhotic liver and hepatocellular carcinoma. Int J Cancer. 1999;81(4):519–26.
Iavarone M, et al. Increased expression of vascular endothelial growth factor in small hepatocellular carcinoma. J Viral Hepat. 2007;14(2):133–9.
Karbaschian Z, et al. Portal and systemic levels of visfatin in morbidly obese subjects undergoing bariatric surgery. Endocrine. 2013;44:114–8.
Koo DJ, et al. Is gut the major source of proinflammatory cytokine release during polymicrobial sepsis? Biochim Biophys Acta. 1999;1454(3):289–95.
Narumi S, et al. Expression of IFN-inducible protein-10 in chronic hepatitis. J Immunol. 1997; 158(11):5536–44.
Nishioji K, et al. Increase of chemokine interferon-inducible protein-10 (IP-10) in the serum of patients with autoimmune liver diseases and increase of its mRNA expression in hepatocytes. Clin Exp Immunol. 2001;123(2):271–9.
Poon RT-P, et al. Quantitative correlation of serum levels and tumor expression of vascular endothelial growth factor in patients with hepatocellular carcinoma. Cancer Res. 2003; 63(12):3121–6.
Porowski D, et al. Liver failure impairs the intrahepatic elimination of Interleukin-6, Tumor Necrosis Factor-alpha, Hepatocyte Growth Factor and Transforming Growth Factor-beta. BioMed Res Int. 2015;934065:1–7.
Qian C, et al. Increased IL-23 and IL-17 expression by peripheral blood cells of patients with primary biliary cirrhosis. Cytokine. 2013;64(1):172–80.
Racanelli V, Rehermann B. The liver as an immunological organ. Hepatology. 2006;43(2 Suppl 1): S54–62.
Ramis JM, et al. Tissue leptin and plasma insulin are associated with lipoprotein lipase activity in severely obese patients. J Nutr Biochem. 2005;16(5):279–85.
Serizawa N, et al. Galectin 3 regulates HCC cell invasion by RhoA and MLCK activation. Lab Invest. 2015;95(10):1145–56.
Sookoian S, et al. Circulating levels and hepatic expression of molecular mediators of atherosclerosis in nonalcoholic fatty liver disease. Atherosclerosis. 2010;209(2):585–91.
Soresi M, et al. Interleukin-6 and its soluble receptor in patients with liver cirrhosis and hepatocellular carcinoma. World J Gastroenterol. 2006;12(16):2563–8.
Van Harmelen V, et al. Leptin secretion from subcutaneous and visceral adipose tissue in women. Diabetes. 1998;47(6):913–7.
Verrijken A, et al. Prothrombotic factors in histologically proven nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Hepatology. 2014;59(1):121–9.
Vonghia L, et al. Peripheral and hepatic vein cytokine levels in correlation with non-alcoholic fatty liver disease (NAFLD)-related metabolic, histological and haemodynamic features. PLoS One. 2015;10(11):e0143380.
Vuppalanchi R, et al. Oxidative stress in chronic liver disease: relationship between peripheral and hepatic measurements. Am J Med Sci. 2013;342(4):314–7.
Wald O, et al. Involvement of the CXCL12/CXCR4 pathway in the advanced liver disease that is associated with hepatitis C virus or hepatitis B virus. Eur J Immunol. 2004;34(4):1164–74.

Wald O, et al. Chemokines in hepatitis C virus infection: pathogenesis, prognosis and therapeutics. Cytokine. 2007;39(1):50–62.
Wanninger J, et al. Systemic and hepatic vein galectin-3 are increased in patients with alcoholic liver cirrhosis and negatively correlate with liver function. Cytokine. 2011;55(3):435–40.
Weigert J, et al. Serum galectin-3 is elevated in obesity and negatively correlates with glycosylated hemoglobin in type 2 diabetes. J Clin Endocrinol Metab. 2010;95(3):1404–11.
Wieckowska A, et al. Increased hepatic and circulating interleukin-6 levels in human nonalcoholic steatohepatitis. Am J Gastroenterol. 2008;103(6):1372–9.
Wiest R, et al. Splanchnic concentrations and postprandial release of visceral adipokines. Metab Clin Exp. 2010;59(5):664–70.
Wiest R, et al. Impaired hepatic removal of interleukin-6 in patients with liver cirrhosis. Cytokine. 2011;53(2):178–83.

Pentraxin 3 (PTX3) as a Biomarker of Liver Disease

29

Bongkun Choi and Eun-Ju Chang

Contents

Abstract

Pentraxins are a family of multifunctional pattern-recognizing proteins that are evolutionarily conserved. Pentraxin 3 (PTX3) – the prototype protein of the long pentraxin group – is a critical component of the humoral arm of innate immunity and opsonic activity, facilitates pathogen recognition, and is produced by a variety of tissues and cells in response to proinflammatory signals and Toll-like receptor engagement. Despite the protective functions of PTX3 that are involved in infection

B. Choi • E.-J. Chang (✉)
Department of Biomedical Sciences, University of Ulsan College of Medicine, Asan Medical Center, Songpa-gu, Seoul, Republic of Korea
e-mail: bkchoi89@hanmail.net; ejchang@amc.seoul.kr

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_32

and female fertility, the persistent elevation in PTX3 levels is reportedly associated with disease severity and increased morbidity in several clinical pathological conditions, including psoriasis, unstable angina pectoris, atherosclerosis, acute myocardial infarction, and ischemic heart disorders. Here, we review the key properties of PTX3 related to liver pathology and discuss recent data suggesting that PTX3 demonstrates liver pathogenic effects. Persistently elevated PTX3 may represent a novel and promising biomarker of liver disease, which correlates with the risk of developing liver injury and pathologic events, thereby providing useful prognostic information for clinical outcomes in patients with this pathological condition.

Keywords

PTX3 • Biomarker • Liver • Inflammation • Nonalcoholic fatty liver disease • Nonalcoholic steatohepatitis

List of Abbreviations

ALF	Acute liver failure
ALT	Alanine aminotransferase
APAP	N-acetyl-p-aminophenol
AST	Aspartate aminotransferase
CKD	Chronic kidney disease
CMV	Cytomegalovirus
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
KO	Knockout
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
OLT	Orthotopic liver transplantation
POD	Paracetamol overdose
PTX3	Pentraxin 3
SAP	Serum amyloid P component
siRNA	Small interfering RNA
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Key Facts

- Pentraxin 3 (PTX3) has a cyclic pentameric structure and was first identified as a cytokine-inducible gene in a variety of cells.
- PTX3 expression is induced by a variety of inflammatory signals such as cytokines, Toll-like receptor (TLR) agonists, microbial moieties (e.g., lipopolysaccharide and outer membrane protein A), and intact microorganisms.
- PTX3 is involved in complement activation, opsonization, and glycosylation-dependent regulation of inflammation.

- An elevated plasma/serum PTX3 concentration is related to various clinical conditions, including bacterial, viral, and fungal infections, autoimmune diseases, cardiovascular diseases, chronic kidney disease, end-stage renal diseases, acute lung injury, pleurisy, endotoxic shock, sepsis, ischemic stroke, subarachnoid hemorrhage, and various tumors.
- Elevated PTX3 concentrations are also related to liver-associated pathological conditions.
- PTX3 is directly produced by damaged tissues, and a rapid increase indicates inflammation.
- In particular, the combination of PTX3 and classic biomarkers provides additional diagnostic and prognostic value to several clinical conditions.

Definitions of Words and Terms

Biomarker	A biomarker is a biological molecule found in the body fluids that is used as a sign of normal or abnormal condition or disease. Biomarkers play an important role in measuring and indicating biological and pathological conditions. Biomarkers also have a special position in understanding the relationships between pathological processes and pathogenesis.
Inflammation	Inflammation is an immune response to infection or irritation. It is characterized by the following quintet: redness, heat, swelling, pain, and dysfunction of the involved organs. Acute inflammation is short lasting and typically only lasts for a few days. If inflammation is longer lasting, however, it is then referred to as chronic inflammation. Chronic inflammation is a prolonged inflammatory response (weeks, months, or even indefinitely), which is prolonged by the persistence of the causative stimulus of the inflammation in the tissue. The inflammatory process unavoidably causes tissue damage and is accompanied by concurrent healing and repair.
Nonalcoholic fatty liver disease	Nonalcoholic fatty liver disease (NAFLD) is an increasingly recognized condition that includes steatosis and nonalcoholic steatohepatitis (NASH) and may progress to fibrosis and cirrhosis. Obesity, type 2 diabetes mellitus, and hyperlipidemia are associated with NAFLD in more than 80% of cases. Other causes of liver disease should be

	excluded, but exact differentiation is often not possible because steatosis and steatohepatitis can sensitize the liver to other pathogens.
Nonalcoholic steatohepatitis	Nonalcoholic steatohepatitis (NASH) is part of the spectrum of nonalcoholic fatty liver diseases (NAFLDs). NAFLD encompasses hepatic lesions such as steatosis, steatohepatitis, fibrosis, cirrhosis, and in some cases hepatocellular carcinoma. NASH mimics alcoholic hepatitis and is histologically defined by the presence of hepatocellular injury (e.g., steatosis, ballooning, cell death), inflammation, and fibrosis in patients consuming up to 20–25 g ethanol daily.
Pathogenesis	Pathogenesis is the mechanism that produces and develops a diseased or morbid condition. There are many different types of pathogenesis, including inflammation, microbial infection, physical trauma, cancerous cells, genetic disorders, and tissue breakdown.
Pentraxins	Pentraxins are a family characterized by the presence of pattern-recognizing proteins in the carboxy-terminal region, which contain a homologous pentraxin domain known as the pentraxin signature. Based on the primary structure of the composing promoters, pentraxins are divided into two groups: short and long pentraxins. The C-reactive protein (CRP) and serum amyloid P component (SAP) are typical short pentraxins. PTX3, NP1, NP2, and PTX4 are typical long pentraxins.

Introduction

Pentraxin 3 (PTX3) – the prototype protein of the long pentraxin group – is an essential component of the humoral arm of innate immunity that demonstrates opsonic activity, thereby facilitating pathogen recognition, and is produced by a variety of tissues and cells in response to proinflammatory signals and Toll-like receptor (TLR) engagement (Alles et al. 1994; Doni et al. 2003). Moreover, PTX3 is a multifunctional protein involved in tuning inflammation, extracellular matrix construction, and female fertility (Garlanda et al. 2005; Jeannin et al. 2005; Bottazzi et al. 2006). Several lines of evidence suggest that PTX3 may be a useful serological marker of tissue inflammation and damage under diverse clinical conditions (Latini et al. 2004; Bevelacqua et al. 2006; Inoue et al. 2007; Suzuki et al. 2008).

Here, we review the key properties of PTX3 as a mediator of liver pathogenesis, with an emphasis on PTX3 as a prototypic member of the long pentraxin family, and recent data suggesting that persistently elevated PTX3 may represent a new and useful biomarker for clinical outcomes in liver pathologic conditions.

Cellular Sources of PTX3

Various types of cell express PTX3, and PTX3 may play a critical role in defense mechanisms. Innate immune cells, such as macrophages and dendritic cells, produce elevated levels of PTX3 in response to stimulation by proinflammatory signals or TLR engagement (Doni et al. 2003), and neutrophils serve as a reservoir for PTX3 proteins that are immediately secreted upon microbial recognition and inflammatory signaling. Moreover, recent studies suggest that PTX3 plays an important role in physiological and pathological function in various organ systems, in addition to its role in innate immunity. Indeed, PTX3 is produced by a variety of tissues, including bone and immune cells.

Liver Tissue Cells

In normal liver tissue, sinusoidal cells with Kupffer cell morphology strongly express PTX3, and biliary epithelial cells moderately express PTX3. While hepatocytes are negative for PTX3 expression in normal liver tissue, membranous PTX3 staining has been observed in the injured hepatocytes of paracetamol explants (Craig et al. 2013). Contrary to hepatocytes, hepatic progenitor cells that have been isolated from the livers of human patients who have undergone partial hepatectomy express PTX3 at levels 20-fold higher than primary hepatocytes and the increased expression of hematopoietic cell markers CD45 and CD109 (Li et al. 2014), thereby suggesting that several cells in the liver tissue at least in part produce PTX3 (Fig. 1).

Other Various Tissue Cells

The classic pentraxins – such as C-reactive protein (CRP) and serum amyloid P component (SAP) – are primarily produced by hepatocytes within the liver in response to primary inflammatory mediators, most prominently IL-6 (Jaillon et al. 2007; Mantovani et al. 2008). Contrary to CRP and SAP, PTX3 is expressed at low levels in the liver, if at all (Lee et al. 1994), and mostly produced locally (not systemically) at the site of inflammation by a wide range of different cell types, including macrophages, myeloid dendritic cells, fibroblasts, vascular endothelial cells, smooth muscle cells, adipocytes, chondrocytes, and epithelial cells (Breviario et al. 1992; Alles et al. 1994; Vouret-Craviari et al. 1997; Abderrahim-Ferkoune et al. 2003; Doni et al. 2003; Klouche et al. 2004; Han et al. 2005; He et al. 2007), and locally exerts its function in response to several stimuli such as proinflammatory signals, Toll-like receptor engagement, tumor necrosis factor (TNF)-α, and IL-1β (Garlanda et al. 2005).

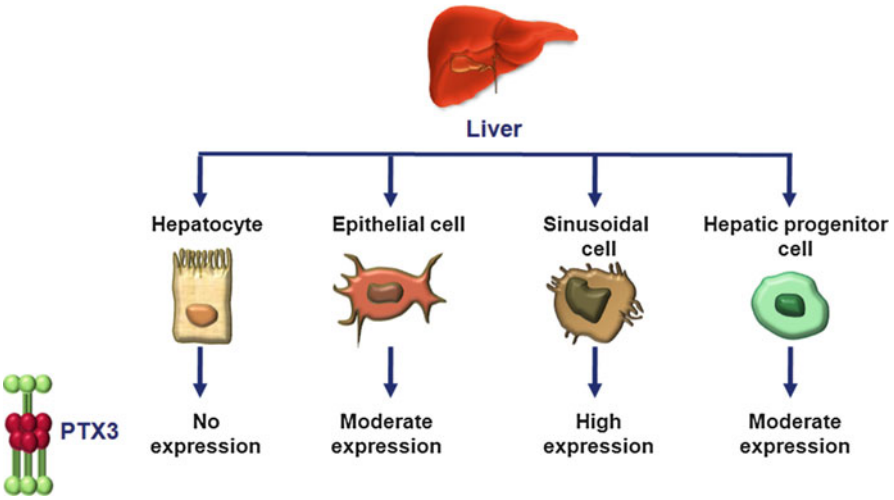

Fig. 1 PTX3 expression in liver tissue cells. In normal liver tissue, hepatocytes are negative for PTX3 expression, but hepatic progenitor cells express PTX3 at levels 20-fold higher than primary hepatocytes. Sinusoidal cells with Kupffer cell morphology strongly express PTX3, and biliary epithelial cells moderately express PTX3

Immune Cells

Neutrophils contain PTX3 at a concentration of approximately 25 ng per 10^6 cells within cytoplasmic granules and promptly release it following degranulation in response to inflammatory signals or infection. These cells thereby serve as a reservoir of PTX3 prior to its biosynthesis by other cell types (Jaillon et al. 2007). Afterward, a prolonged elevation of PTX3 arises from its induced expression by monocytes, macrophages, and dendritic cells (Alles et al. 1994; Doni et al. 2003). For example, dendritic cells can secrete approximately 50 ng PTX3 protein per 10^6 cells over a period of 24 h (Doni et al. 2003). In patients with acute liver failure (ALF), the infiltrating inflammatory mononuclear cells are strongly positive for PTX3 expression in liver tissue (Craig et al. 2013).

PTX3 as a Biomarker of Acute Liver Injury

Analgesic Overdose and Drug-Induced Liver Injury

Acute liver failure (ALF) is a devastating clinical syndrome characterized by hepatic encephalopathy, coagulopathy, and high mortality caused by high infection rates (Larson et al. 2005). Dysfunction of the cellular arm of the innate immune system

has been suggested as a mechanism responsible for the increased risk of infection in ALF patients. However, the role of the humoral components of innate immunity in ALF is not well elucidated. Although elevated levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are indicative of hepatocellular damage, overall these enzymes are poor prognostic markers of the severity of liver injury or ALF (Huang et al. 2008).

Overdosing with an over-the-counter analgesic and antipyretic drug – such as acetaminophen (N-acetyl-p-aminophenol [APAP]) (Bessems et al. 2011) – often leads to severe liver injury in humans and experimental animals (O'Grady 1997) and is one of the most common etiologies of ALF. The characteristic features of APAP-induced hepatotoxicity are high degrees of necrosis and the infiltration of proinflammatory cells into the centrilobular regions of the liver. Administering APAP-1 and APAP-2 to adult male Wistar rats causes hepatic injury, including hepatocyte necrosis on histopathological examination and elevated liver PTX3 levels in comparison with control groups ($p < 0.001$ and $p < 0.001$, respectively) (Yaman et al. 2013). Cellular PTX3 levels in the livers of rats in the APAP-1 and APAP-2 groups were dramatically elevated to 14.1 ± 3.0 ng/mg protein ($p = 0.032$) and 28.5 ± 8.2 ng/mg protein ($p < 0.001$), respectively, in comparison with the control group (7.5 ± 3.3 ng/mg protein), although ASAP-2 ($p < 0.001$) but not ASAP-1 treatment ($p = 0.135$) significantly increases the plasma PTX3 level (Yaman et al. 2013). Thus, elevated liver PTX3 in APAP-induced hepatic necrosis is a potential biomarker of the primary local activation of inflammation in APAP-induced, acute, histological liver injury (Yaman et al. 2013).

Craig et al. reported that the median admission plasma PTX3 level was significantly higher in paracetamol-overdose (POD) patients (148.6 ng/ml [range = 26.6–579]; $n = 48$) in comparison with non-POD patients (23.7 ng/ml [range = 9.1–40.0]; $n = 12$; $p = 0.004$), which correlated with IL-6, IL-10, and multiorgan failure (Craig et al. 2013). Moreover, the PTX3 levels in POD patients who developed systemic inflammatory response syndrome required orthotopic liver transplantation (OLT) or died (568.2 ng/ml [range = 76.4–832.7]; $n = 14$) were significantly higher in comparison with those measured in spontaneous survivors (64.3 ng/ml [range = 10.0–372.3]; $n = 34$; $p = 0.0011$), and the sinusoidal lining cells of normal liver tissue and the adjacent infiltrating inflammatory immune cells strongly expressed PTX3 in patients with ALF (Craig et al. 2013). In contrast, the CRP levels were significantly lower in POD patients (6.05 mg/l [range = 3.93–15.38]; $n = 48$] in comparison with non-POD patients (17.55 mg/l [range = 3.93–15.38]; $n = 12$; $p = 0.011$) (Craig et al. 2013). Moreover, removing the injured liver followed by orthotopic liver transplantation led to a rapid and marked decline in circulating PTX3 levels but a rapid increase in plasma CRP levels within 3–4 days (Craig et al. 2013). These results suggest that PTX3 – the component of the humoral arm of innate immunity – may be involved in ALF pathogenesis. Further studies need to evaluate the prognostic value of PTX3 for predicting histological hepatic necrosis in acute liver injury.

PTX3 in Nonalcoholic Fatty Liver Disease

Novel Marker for Nonalcoholic Steatohepatitis (NASH)

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of abnormal liver function, resulting in chronic liver injury that is characterized by the accumulation of large triglyceride droplets within hepatocytes, and no specific treatment for NAFLD is available (Erickson 2009). This excess storage of fat in the liver sensitizes the organ to inflammation and fibrosis, subsequently leading to macrovesicular hepatic steatosis (Angulo 2002). NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis, and cirrhosis (Farrell and Larter 2006), and several clinical risk factors have been proposed for liver disease and NAFLD including old age, type 2 diabetes mellitus, obesity, and cardiovascular disease (Ratziu et al. 2000; Shimada et al. 2002; Sakugawa et al. 2005; Yoneda et al. 2007) (Table 1). While simple steatosis without fibrosis or inflammation demonstrates a benign clinical course (Ekstedt et al. 2006), NASH demonstrates a more progressive course that leads to cirrhosis (Xie et al. 2012) and lower survival rate (Ekstedt et al. 2006; Soderberg et al. 2010). It is therefore important to distinguish simple steatosis from NASH in order to intervene and slow down disease progression in NASH patients.

Liver biopsy is still considered the gold standard for evaluating both the diagnosis and staging in NASH patients; however, the limitations of liver biopsy include its level of invasiveness, cost, and potential to cause rare sampling errors (Ratziu et al. 2005; Younossi et al. 2008) and damage to the biliary system (Baranova et al. 2011). Hence, it is not suitable as a screening method for a condition that affects almost 30% of the population in the United States, and alternative noninvasive methods that would help avoid liver biopsy are needed (Wieckowska et al. 2007). Therefore, noninvasive biomarkers that are cheaper, more reliable,

Table 1 Clinical risk factors of fibrosis in NAFLD patients and severe liver injuries

	Disease	Disease characteristics
Paracetamol overdose	Acute liver injury	Hepatic encephalopathy, coagulopathy, and high infection rates
Acetaminophen (analgesic drug)	Severe liver injury	Accumulation of macrophages in the liver
Old age	NAFLD	
Type 2 diabetes mellitus	NAFLD	
Obesity	NAFLD	Chronic and subacute inflammation, 57.5–74% NAFLD in obese population
Cardiovascular disease	NAFLD	
Acinetobacter baumannii	Septic shock	Neutrophil recruitment, severe liver pathology, and coagulopathy
Cytomegalovirus (CMV)	Liver transplantation	

NAFLD nonalcoholic fatty liver disease

and reproducible are urgently required for patients with NASH in order to establish diagnoses and monitor disease progression and treatment response.

In this respect, various noninvasive methods such as imaging studies and blood markers have been applied for definite diagnosis, but these tools are not standardized for the accurate diagnosis of the severity of liver fibrosis (Cales et al. 2005; Festi et al. 2013). Imaging studies, including ultrasonography, computed tomography, elastography, and magnetic resonance imaging, demonstrate limited sensitivity and specificity for detecting steatosis and distinguishing between simple steatosis and NASH, although these methods are noninvasive and can be repetitively performed over a period of time (Wieckowska et al. 2007).

There is growing evidence regarding the correlation between elevated levels of serum biomarkers, NAFLD severity, and liver injuries. Several diagnostic panels have been developed to predict significant liver disease and fibrosis. Different diagnostic panels and several serum markers that are correlated with NAFLD and liver injuries have been suggested, including serum transaminase level, platelet count, and high-sensitivity C-reactive protein (CRP) (Ratziu et al. 2000; Shimada et al. 2002; Sakugawa et al. 2005; Yoneda et al. 2007), which are summarized in Table 2. However, the strongly positive role of serum biomarkers for evaluating the severity of liver fibrosis has not been established. Although alanine aminotransferase (ALT) has been used as a surrogate marker for liver injury, it is not an ideal biomarker for either diagnosing NAFLD or distinguishing steatosis from NASH (Mofrad et al. 2003; Browning et al. 2004; Sorrentino et al. 2004).

Yoneda et al. reported that PTX3 levels are closely associated with the stages of hepatic fibrosis, and the plasma PTX3 levels in patients with stage 3–4 nonalcoholic fatty liver disease (NAFLD) are profoundly elevated in comparison with patients with stage 0–2 NAFLD ($p < 0.0001$) (Yoneda et al. 2008), thereby indicating that PTX3 levels are strongly correlated with disease severity. In addition, the plasma PTX3 level was significantly higher in NASH patients than non-NASH patients ($p = 0.0021$) or healthy controls ($p = 0.045$) (Yoneda et al. 2008), thereby suggesting that plasma PTX3 may be useful for targeted therapies against fibrosis in NASH patients.

The administration of coenzyme Q10, a potent antioxidant, leads to a decline in hepatic oxidative stress and inflammation in a diet-induced obese rat model (Sohet et al. 2009), and serum PTX3 concentrations decreased in the coenzyme Q10-treated group (Farhangi et al. 2014). However, the precise mechanism underlying the upregulation of PTX3 is unknown, and future studies are needed.

PTX3 in Infection Associated with Liver Pathology and Liver Transplant

Sepsis

PTX3 recognizes and interacts with a variety of pathogens, such as bacteria, fungi, and viruses, eliciting protection against these pathogens by promoting phagocytosis and pathogen clearance (Mantovani et al. 2008). Specific interactions have been

Table 2 Serum markers correlated with NAFLD and liver injuries

Diagnosis and biomarkers	Diagnosis of disease		Cutoff value	AUROC	Se (%)	Sp (%)	Reference
Alanine aminotransferase (ALT), aspartate aminotransferase (AST)	Liver damage	Not ideal for diagnosis of NAFLD or NASH					
Serum transaminase	Severe fibrosis in NAFLD						
Platelet count	NAFLD						
Tumor necrosis factor alpha (TNF-α)	Higher level of TNF-α mRNA in NASH		100 ng/ml TNF-α mRNA	0.685	66.7	74.1	Alaaeddine et al. (2012)
Interleukin 6 (IL-6)	Higher plasma level of IL-6 in NASH		4.6 ng/ml IL-6	0.817	58.1	100	Tarantino et al. (2009)
High-sensitivity C-reactive protein (CRP)	Useful for diagnosis of hepatic steatosis or NASH	Not a good predictor of severe fibrosis or severity of NAFLD	3.5 mg/ml CRP	0.906	82	88	Fierbinteanu-Braticevici et al. (2011)
Pentraxin 3 (PTX3)	(Acute) liver injury		1.61 ng/ml PTX3	0.755	66.7	78.6	Yoneda et al. (2008)
	NASH from simple steatosis						
	Distinguishing stages 3–4 from stages 0 to 2 NAFLD		2.45 ng/ml PTX3	0.85	70.6	94.3	

Ferritin	NASH from simple steatosis	Not predicting the stage of NAFLD	240 ng/ml ferritin	0.732	64.2	76.5	Yoneda et al. (2010)
				0.82	91	70	Manousou et al. (2011)
Constituents of extracellular matrix (ECM)	NASH from simple steatosis		≥148.8 ng/ml hyaluronic acid	0.975	97.5	95	Lydatakis et al. (2006)
Apoptosis markers, cytokeratin 18 (CK18)	NASH from simple steatosis		395 U/L CK18	0.93	85.7	99.9	Wieckowska et al. (2006)
			243.8 U/L CK18	0.787	68.9	81.6	Yilmaz et al. (2007)

NAFLD nonalcoholic fatty liver disease, *NASH* nonalcoholic steatohepatitis, *AUROC* area under receiver operator characteristic curve, *Se* sensitivity, *Sp* specificity

observed with several gram-positive and gram-negative bacteria, including *Klebsiella pneumoniae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (Jeannin et al. 2005; Deban et al. 2011; Moalli et al. 2011; Paroni et al. 2013). Consistent with its binding to bacteria, PTX3 also binds to *Paracoccidioides brasiliensis* and conidia from *Aspergillus fumigatus* (Garlanda et al. 2002) and recognizes viruses such as cytomegalovirus and influenza virus type A (Bozza et al. 2006), thereby suggesting that PTX3 plays a role in the recognition and resistance against specific pathogens.

The PTX3 stored in neutrophil granules is rapidly released in response to inflammatory signals and partially localizes in neutrophil extracellular traps, where it exhibits opsonic activity that facilitates the phagocytosis and killing of *Aspergillus fumigatus* (Jaillon et al. 2007). The risk factors for invasive aspergillosis in patients undergoing chemotherapy or transplant have been identified, and patients undergoing liver transplant due to hepatic failure are also susceptible to invasive aspergillosis (Marr et al. 2002; Singh and Husain 2013). PTX3 released from neutrophils and produced by various cells recognizes microbial moieties and opsonizes pathogens in order to enhance complement, thereby facilitating pathogen recognition, phagocytosis, and pathogen clearance by stimulating the Fcχ receptor, recruiting the C1q complement component, and facilitating innate immune cell activation including elevated cytokine and nitric oxide production (Garlanda et al. 2002; Nauta et al. 2003; Deban et al. 2010, 2011; Moalli et al. 2010). Despite the protective functions of PTX3 that are involved in infection, female fertility, and myocardial infarction (Garlanda et al. 2002; Moalli et al. 2010; Deban et al. 2011), the persistent elevation of patient PTX3 levels is reportedly associated with disease severity and increased morbidity in severe sepsis (Muller et al. 2001; Sprong et al. 2009; Wagenaar et al. 2009; Mauri et al. 2010; Huttunen et al. 2011; Vanska et al. 2011; Uusitalo-Seppala et al. 2013).

PTX3 functions as an acute-phase protein, and its level in the blood rapidly increases, reaching a peak in 6–8 h in comparison with 36–48 h for CRP, and also dramatically increases from <2 ng/ml under normal conditions to 200–800 ng/ml, which is followed by endotoxic shock, sepsis, and other inflammatory or infectious conditions (Muller et al. 2001; Azzurri et al. 2005; Mairuhu et al. 2005; Sprong et al. 2009; Wagenaar et al. 2009; Mauri et al. 2010). In all cases, the PTX3 plasma levels are significantly correlated with disease severity (Sprong et al. 2009).

Acinetobacter baumannii is one of the most prevalent bacterial pathogens associated with human traumatic wounds and septic shock with bloodstream infections (Kang et al. 2010). *A. baumannii* infection leads to neutrophil recruitment and activation accompanied with the elevation of the leukocyte chemokines CXCL1, CCL2, and CCL5 (Ketter et al. 2014). Severe *A. baumannii* sepsis is associated with the significant and rapid elevation in serum PTX3 levels (10-fold elevation), which peaks at 12 h post-challenge and maintains sustained levels of PTX3 over 24 h post-challenge in a mouse model (Ketter et al. 2014). Elevated PTX3 is accompanied by severe pathology in the liver, but not in the spleen or kidney, as evidenced by inflammatory foci in the liver with polymorphonuclear cell infiltration, distinct

apoptotic nuclei, and hypercoagulopathy represented by vessel blockage and tissue damage; these findings suggest that the prolonged elevation of PTX3 during *A. baumannii* sepsis is associated with increased disease severity (Ketter et al. 2014).

Cytomegalovirus

Cytomegalovirus (CMV) is one of the most common viral pathogens that influence the outcomes of liver transplantation. CMV is associated with increased allograft rejection, accelerated hepatitis C recurrence, other opportunistic infections, and reduced overall patient survival (Razonable 2008). Solid organ transplant recipients who fail to suppress CMV replication also demonstrate significantly higher baseline levels of PTX3 in comparison with patients who achieve lower viral loads ($p < 0.001$) (Rollag et al. 2012).

Potential Applications of Predicting Prognosis and Other Diseases or Conditions

Several lines of evidence suggest that measuring PTX3 in the blood can be applied to the early diagnosis and prognosis of several diseases such as psoriasis (Bevelacqua et al. 2006), vascular pathologies such as unstable angina pectoris (Inoue et al. 2007), acute myocardial infarction (Peri et al. 2000; Latini et al. 2004), atherosclerosis (Zanetti et al. 2009), and ischemic heart disorders (Suzuki et al. 2008), thereby suggesting that PTX3 could be a prognostic marker of heart disorders. PTX3 is involved in the mechanisms underlying the development of atherosclerosis (Alberti et al. 2009), as the macrophages and polymorphonuclear cells that infiltrate atherosclerotic plaques are positive for PTX3 (Savchenko et al. 2008). In particular, highly elevated circulating levels of PTX3 were observed in patients with arterial inflammation, unstable angina pectoris (Inoue et al. 2007), coronary artery disease (Kotooka et al. 2008), and acute myocardial infarction (Peri et al. 2000; Latini et al. 2004), suggesting that PTX3 may be a useful marker for evaluating the inflammatory responses to vascular pathology.

Obesity accompanies chronic systemic inflammation and is one of the strongest risk factors for liver diseases and cardiovascular mortality. Blood PTX3 levels are substantially higher in obese men (0.99 ± 0.09 ng/ml) than nonobese controls (0.63 ± 0.05 ng/ml) (Miyaki et al. 2010) and also elevated in patients with metabolic syndrome (Zanetti et al. 2009). Autoimmune disorders such as small vessel vasculitis, systemic sclerosis, and rheumatoid arthritis, but not systemic lupus erythematosus, exhibit increased levels of PTX3 (Luchetti et al. 2000; Fazzini et al. 2001; Shirai et al. 2015) which correlates with the clinical activity (Fazzini et al. 2001). Sepsis, endotoxic shock, and other infectious conditions also lead to the rapid and dramatic elevation in PTX3 expression (Nauta et al. 2003; Razonable 2008; Kang et al. 2010).

In addition, patients with chronic kidney disease (CKD) demonstrate significant increases in circulating PTX3 levels with correlations between the PTX3 plasma concentration and disease severity, thereby suggesting that PTX3 could predict mortality in CKD patients (Tong et al. 2007). Moreover, several reports show a slight increase in maternal circulating PTX3 levels during pregnancy, and complications such as preeclampsia, spontaneous preterm delivery, and placenta vasculopathy further increase PTX3 concentrations due to excessive maternal inflammatory responses that occurs during pregnancy (Cetin et al. 2006; Assi et al. 2007). Thus, PTX3 in association with classic biomarkers may provide diagnostic and prognostic value toward determining the clinical outcomes of these pathologic conditions.

The presence of PTX3 in bone-related cells and the role of PTX3 in bone-associated diseases have not been well elucidated. Osteoblasts derived from bone marrow stromal cells highly express PTX3. PTX3 induces production of receptor activator of NFkB ligand (RANKL) from osteoblasts, thereby contributing to the osteolysis as an inflammatory mediator in the bone environment (Lee et al. 2014). Moreover, PTX3 expression is elevated in the distant bone metastases of breast cancer, and PTX3 plays a key role in the inflammation-associated osteolytic complications of breast cancer (Choi et al. 2014). Recent data suggests that persistently elevated PTX3 as a mediator of bone pathogenesis may represent a new and useful biomarker for clinical outcomes in bone pathologic condition (Choi et al. 2016).

Conclusion

We have here focused on the role of PTX3 as a mediator and marker of liver pathology. The expression levels of PTX3 in tissue are elevated in response to overdoses of analgesic drugs, microbes (*Acinetobacter baumannii* and cytomegalovirus), and diverse diseases, thereby leading to liver injury and pathology; therefore, elevated PTX3 levels may function as a potential serological biomarker of liver pathology (Fig. 2). It has also been suggested that PTX3 measurement and, in particular, the combination of PTX3 and classic biomarkers in the blood can be applied to the early diagnosis and prediction of the prognosis of several diseases such as psoriasis (Bevelacqua et al. 2006), unstable angina pectoris (Inoue et al. 2007), acute myocardial infarction (Latini et al. 2004), and heart failure (Suzuki et al. 2008). The general characteristics of the PTX3 levels in the blood in human pathologies include the rapid increase in PTX3 in comparison with CRP (Breviario et al. 1992; Peri et al. 2000) and the lack of correlation between PTX3 levels and CRP levels. Likewise, PTX3 levels are correlated with liver disease activity and severity, and thus PTX3 could be a biomarker in combination with other serological biomarkers for the noninvasive diagnosis of liver disease. Several lines of evidence suggest that PTX3 may be a novel promising biomarker that could provide useful prognostic information regarding

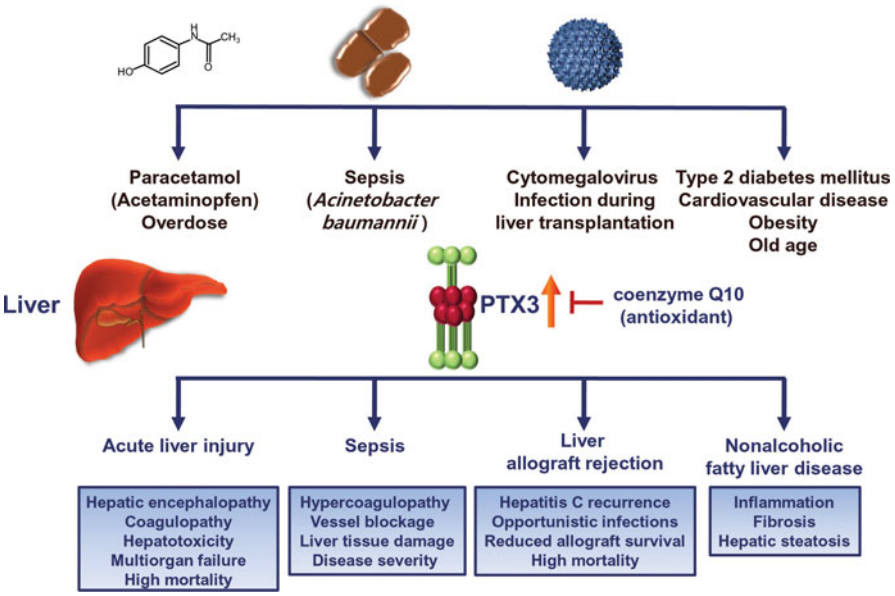

Fig. 2 PTX3 in liver pathology. PTX3 expression is elevated in tissues in response to overdoses of analgesic drugs, microbial sensing, and diverse diseases that lead to liver injury and pathology. Antioxidant treatment leads to declines in PTX3 levels, and elevated PTX3 may function as potential serological biomarker of liver pathology

the clinical outcomes of these pathologic conditions. Future studies need to examine the role of PTX3 and verify the value of PTX3 as a biomarker of clinical liver pathologic events.

Summary Points

- This chapter focuses on the key properties of pentraxin 3 (PTX3) in relation to liver pathology and its potential role as a biomarker of this disease.
- Recently, a number of studies have reported that PTX3 may function as a screening device for inflammation, a marker for disease activity, and as a diagnostic biomarker for the pathogenesis of various human pathologies.
- PTX3 expression is elevated in liver-associated pathological conditions.
- Analgesic drug-induced acute liver injury leads to elevation of PTX3 level.
- PTX3 expression is profoundly elevated in nonalcoholic steatohepatitis patients.
- Elevated PTX3 expression is involved with infection associated with liver pathology and liver transplant.
- PTX3 may be a novel, promising biomarker that provides useful prognostic information on the clinical outcomes in patients with liver pathological conditions.

References

Abderrahim-Ferkoune A, Bezy O, Chiellini C, et al. Characterization of the long pentraxin PTX3 as a TNFalpha-induced secreted protein of adipose cells. J Lipid Res. 2003;44:994–1000.

Alaaeddine N, Sidaoui J, Hilal G, et al. TNF-alpha messenger ribonucleic acid (mRNA) in patients with nonalcoholic steatohepatitis. Eur Cytokine Netw. 2012;23:107–11.

Alberti L, Gilardini L, Zulian A, et al. Expression of long pentraxin PTX3 in human adipose tissue and its relation with cardiovascular risk factors. Atherosclerosis. 2009;202:455–60.

Alles V, Bottazzi B, Peri G, et al. Inducible expression of PTX3, a new member of the pentraxin family, in human mononuclear phagocytes. Blood. 1994;84:3483–93.

Angulo P. Nonalcoholic fatty liver disease. N Engl J Med. 2002;346:1221–31.

Assi F, Fruscio R, Bonardi C, et al. Pentraxin 3 in plasma and vaginal fluid in women with preterm delivery. BJOG. 2007;114:143–7.

Azzurri A, Sow O, Amedei A, et al. IFN-gamma-inducible protein 10 and pentraxin 3 plasma levels are tools for monitoring inflammation and disease activity in Mycobacterium tuberculosis infection. Microbes Infect. 2005;7:1–8.

Baranova A, Lal P, Birerdinc A, et al. Non-invasive markers for hepatic fibrosis. BMC Gastroenterol. 2011;11:91.

Bessems JG, Vermeulen NP. Paracetamol (acetaminophen)-induced toxicity: molecular and biochemical mechanisms, analogues and protective approaches. Crit Rev Toxicol. 2011; 31:55–138.

Bevelacqua V, Libra M, Mazzarino MC, et al. Long pentraxin 3: a marker of inflammation in untreated psoriatic patients. Int J Mol Med. 2006;18:415–23.

Bottazzi B, Garlanda C, Salvatori G, et al. Pentraxins as a key component of innate immunity. Curr Opin Immunol. 2006;18:10–5.

Bozza S, Bistoni F, Gaziano R, et al. Pentraxin 3 protects from MCMV infection and reactivation through TLR sensing pathways leading to IRF3 activation. Blood. 2006;108:3387–96.

Breviario F, d'Aniello E, Golay J, et al. Interleukin-1-inducible genes in endothelial cells. Cloning of a new gene related to C-reactive protein and serum amyloid P component. J Biol Chem. 1992;267:22190–7.

Browning J, Szczepaniak L, Dobbins R, et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology. 2004;40:1387–95.

Cales P, Oberti F, Michalak S, et al. A novel panel of blood markers to assess the degree of liver fibrosis. Hepatology. 2005;42:1373–81.

Cetin I, Cozzi V, Pasqualini F, et al. Elevated maternal levels of the long pentraxin 3 (PTX3) in preeclampsia and intrauterine growth restriction. Am J Obstet Gynecol. 2006; 194:1347–53.

Choi B, Lee EJ, Song DH, et al. Elevated Pentraxin 3 in bone metastatic breast cancer is correlated with osteolytic function. Oncotarget. 2014;5:481–92.

Choi B, Chang EJ. Biomarkers in disease: methods, discoveries and applications. Biomarkers in bone disease. In: Patel V, Preedy V, (eds). Pentraxin 3 (PTX3) as a biomarker of bone disease. Dordrecht: Springer Science+Business Media; 2016.

Craig D, Lee P, Pryde E, et al. Elevated levels of the long pentraxin 3 in paracetamol-induced human acute liver injury. Eur J Gastroenterol Hepatol. 2013;25:359–67.

Deban L, Russo RC, Sironi M, et al. Regulation of leukocyte recruitment by the long pentraxin PTX3. Nat Immunol. 2010;11:328–34.

Deban L, Jaillon S, Garlanda C, et al. Pentraxins in innate immunity: lessons from PTX3. Cell Tissue Res. 2011;343:237–49.

Doni A, Peri G, Chieppa M, et al. Production of the soluble pattern recognition receptor PTX3 by myeloid, but not plasmacytoid, dendritic cells. Eur J Immunol. 2003;33:2886–93.

Ekstedt M, Franzen LE, Mathiesen UL, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology. 2006;44:865–73.

Erickson SK. Nonalcoholic fatty liver disease. J Lipid Res. 2009;50:S412–6.

Farhangi MA, Alipour B, Jafarvand E, et al. Oral coenzyme Q10 supplementation in patients with nonalcoholic fatty liver disease: effects on serum vaspin, chemerin, pentraxin 3, insulin resistance and oxidative stress. Arch Med Res. 2014;45:589–95.
Farrell GC, Larter CG. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. Hepatology. 2006;43:S99–112.
Fazzini F, Peri G, Doni A, et al. PTX3 in small-vessel vasculitides: an independent indicator of disease activity produced at sites of inflammation. Arthritis Rheum. 2001;44:2841–50.
Festi D, Schiumerini R, Marzi L, et al. Review article: the diagnosis of non-alcoholic fatty liver disease – availability and accuracy of non-invasive methods. Aliment Pharmacol Ther. 2013;37:392–400.
Fierbinteanu-Braticevici C, Baicus C, Tribus L, et al. Predictive factors for nonalcoholic steatohepatitis (NASH) in patients with nonalcoholic fatty liver disease (NAFLD). J Gastrointestin Liver Dis. 2011;20:153–9.
Garlanda C, Hirsch E, Bozza S, et al. Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. Nature. 2002;420:182–6.
Garlanda C, Bottazzi B, Bastone A, et al. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. Annu Rev Immunol. 2005;23:337–66.
Han B, Mura M, Andrade CF, et al. TNFalpha-induced long pentraxin PTX3 expression in human lung epithelial cells via JNK. J Immunol. 2005;175:8303–11.
He X, Han B, Liu M. Long pentraxin 3 in pulmonary infection and acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2007;292:L1039–49.
Huang L, Heinloth AN, Zeng ZB, et al. Genes related to apoptosis predict necrosis of the liver as a phenotype observed in rats exposed to a compendium of hepatotoxicants. BMC Genomics. 2008;9:288.
Huttunen R, Hurme M, Aittoniemi J, et al. High plasma level of long pentraxin 3 (PTX3) is associated with fatal disease in bacteremic patients: a prospective cohort study. PLoS One. 2011;6:e17653.
Inoue K, Sugiyama A, Reid PC, et al. Establishment of a high sensitivity plasma assay for human pentraxin3 as a marker for unstable angina pectoris. Arterioscler Thromb Vasc Biol. 2007;27:161–7.
Jaillon S, Peri G, Delneste Y, et al. The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. J Exp Med. 2007;204:793–804.
Jeannin P, Bottazzi B, Sironi M, et al. Complexity and complementarity of outer membrane protein A recognition by cellular and humoral innate immunity receptors. Immunity. 2005;22:551–60.
Kang G, Hartzell JD, Howard R, et al. Mortality associated with Acinetobacter baumannii complex bacteremia among patients with war-related trauma. Infect Control Hosp Epidemiol. 2010;31:92–4.
Ketter PM, Guentzel MN, Schaffer B, et al. Severe Acinetobacter baumannii sepsis is associated with elevation of pentraxin 3. Infect Immun. 2014;82:3910–8.
Klouche M, Peri G, Knabbe C, et al. Modified atherogenic lipoproteins induce expression of pentraxin-3 by human vascular smooth muscle cells. Atherosclerosis. 2004;175:221–8.
Kotooka N, Inoue T, Fujimatsu D, et al. Pentraxin3 is a novel marker for stent-induced inflammation and neointimal thickening. Atherosclerosis. 2008;197:368–74.
Larson AM, Polson J, Fontana RL, et al. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. Hepatology. 2005;42:1364–72.
Latini R, Maggioni AP, Peri G, et al. Prognostic significance of the long pentraxin PTX3 in acute myocardial infarction. Circulation. 2004;110:2349–54.
Lee GW, Goodman AR, Lee TH, et al. Relationship of TSG-14 protein to the pentraxin family of major acute phase proteins. J Immunol. 1994;153:3700–7.
Lee EJ, Song DH, Kim YJ, et al. PTX3 stimulates osteoclastogenesis by increasing osteoblast RANKL production. J Cell Physiol. 2014;229:1744–52.
Li J, Xin J, Zhang L, et al. Human hepatic progenitor cells express hematopoietic cell markers CD45 and CD109. Int J Med Sci. 2014;11:65–79.

Luchetti MM, Piccinini G, Mantovani A, et al. Expression and production of the long pentraxin PTX3 in rheumatoid arthritis (RA). Clin Exp Immunol. 2000;119:196–202.
Lydatakis H, Hager I, Kostadelou E, et al. Non-invasive markers to predict the liver fibrosis in non-alcoholic fatty liver disease. Liver Int. 2006;26:864–71.
Mairuhu A, Peri G, Setiati T, et al. Elevated plasma levels of the long pentraxin, pentraxin 3, in severe dengue virus infections. J Med Virol. 2005;76:547–52.
Manousou P, Kalambokis G, Grillo F, et al. Serum ferritin is a discriminant marker for both fibrosis and inflammation in histologically proven non-alcoholic fatty liver disease patients. Liver Int. 2011;31:730–9.
Mantovani A, Garlanda C, Doni A, et al. Pentraxins in innate immunity: from C-reactive protein to the long pentraxin PTX3. J Clin Immunol. 2008;28:1–13.
Marr KA, Carter RA, Boeckh M, et al. Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. Blood. 2002;100:4358–66.
Mauri T, Bellani G, Patroniti N, et al. Persisting high levels of plasma pentraxin 3 over the first days after severe sepsis and septic shock onset are associated with mortality. Intensive Care Med. 2010;36:621–9.
Miyaki A, Maeda S, Yoshizawa M, et al. Is pentraxin 3 involved in obesity-induced decrease in arterial distensibility? J Atheroscler Thromb. 2010;17:278–84.
Moalli F, Doni A, Deban L. Role of complement and Fc{gamma} receptors in the protective activity of the long pentraxin PTX3 against Aspergillus fumigatus. Blood. 2010;116:5170–80.
Moalli F, Paroni M, Rodriguez TV, et al. The therapeutic potential of the humoral pattern recognition molecule PTX3 in chronic lung infection caused by Pseudomonas aeruginosa. J Immunol. 2011;186:5425–34.
Mofrad P, Contos M, Haque M, et al. Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. Hepatology. 2003;37:1286–92.
Muller B, Peri G, Doni A, et al. Circulating levels of the long pentraxin PTX3 correlate with severity of infection in critically ill patients. Crit Care Med. 2001;29:1404–7.
Nauta A, Bottazzi B, Mantovani A, et al. Biochemical and functional characterization of the interaction between pentraxin 3 and C1q. Eur J Immunol. 2003;33:465–73.
O'Grady JG. Paracetamol-induced acute liver failure: prevention and management. J Hepatol. 1997;26 Suppl 1:41–6.
Paroni M, Moalli F, Nebuloni M, et al. Response of CFTR-deficient mice to long-term chronic Pseudomonas aeruginosa infection and PTX3 therapy. J Infect Dis. 2013;208:130–8.
Peri G, Introna M, Corradi D, et al. PTX3, A prototypical long pentraxin, is an early indicator of acute myocardial infarction in humans. Circulation. 2000;102:636–41.
Ratziu V, Giral P, Charlotte F, et al. Liver fibrosis in overweight patients. Gastroenterology. 2000;118:1117–23.
Ratziu V, Charlotte F, Heurtier A, et al. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. Gastroenterology. 2005;128:1898–906.
Razonable RR. Cytomegalovirus infection after liver transplantation: current concepts and challenges. World J Gastroenterol. 2008;14:4849–60.
Rollag H, Asberg A, Ueland T, et al. Treatment of cytomegalovirus disease in solid organ transplant recipients: markers of inflammation as predictors of outcome. Transplantation. 2012;94:1060–5.
Sakugawa H, Nakayoshi T, Kobashigawa K, et al. Clinical usefulness of biochemical markers of liver fibrosis in patients with nonalcoholic fatty liver disease. World J Gastroenterol. 2005;11:255–9.
Savchenko A, Imamura M, Ohashi R, et al. Expression of pentraxin 3 (PTX3) in human atherosclerotic lesions. J Pathol. 2008;215:48–55.
Shimada M, Hashimoto E, Kaneda H, et al. Nonalcoholic steatohepatitis: risk factors for liver fibrosis. Hepatol Res. 2002;24:429–38.
Shirai Y, Okazaki Y, Inoue Y, et al. Elevated levels of pentraxin 3 in systemic sclerosis: associations with vascular manifestations and defective vasculogenesis. Arthritis Rheumatol. 2015;67:498–507.

Singh N, Husain S. Aspergillosis in solid organ transplantation. Am J Transplant. 2013;13 Suppl 4:228–41.
Soderberg C, Stal P, Askling J, et al. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. Hepatology. 2010;51:595–602.
Sohet FM, Neyrinck AM, Pachikian BD, et al. Coenzyme Q10 supplementation lowers hepatic oxidative stress and inflammation associated with diet-induced obesity in mice. Biochem Pharmacol. 2009;78:1391–400.
Sorrentino P, Tarantino G, Conca P, et al. Silent non-alcoholic fatty liver disease-a clinical-histological study. J Hepatol. 2004;41:751–7.
Sprong T, Peri G, Neeleman C, et al. Pentraxin 3 and C-reactive protein in severe meningococcal disease. Shock. 2009;31:28–32.
Suzuki S, Takeishi Y, Niizeki TY, et al. Pentraxin 3, a new marker for vascular inflammation, predicts adverse clinical outcomes in patients with heart failure. Am Heart J. 2008;155:75–81.
Tarantino G, Conca P, Pasanisi F, et al. Could inflammatory markers help diagnose nonalcoholic steatohepatitis? Eur J Gastroenterol Hepatol. 2009;21:504–11.
Tong M, Carrero JJ, Qureshi AR. Plasma pentraxin 3 in patients with chronic kidney disease: associations with renal function, protein-energy wasting, cardiovascular disease, and mortality. Clin J Am Soc Nephrol. 2007;2:889–97.
Uusitalo-Seppala R, Huttunen R, Aittoniemi J, et al. Pentraxin 3 (PTX3) is associated with severe sepsis and fatal disease in emergency room patients with suspected infection: a prospective cohort study. PLoS One. 2013;8:e53661.
Vanska M, Koivula I, Hamalainen S, et al. High pentraxin 3 level predicts septic shock and bacteremia at the onset of febrile neutropenia after intensive chemotherapy of hematologic patients. Haematologica. 2011;96:1385–9.
Vouret-Craviari V, Matteucci C, Peri G, et al. Expression of a long pentraxin, PTX3, by monocytes exposed to the mycobacterial cell wall component lipoarabinomannan. Infect Immun. 1997;65:1345–50.
Wagenaar JF, Goris MG, Gasem MH, et al. Long pentraxin PTX3 is associated with mortality and disease severity in severe Leptospirosis. J Infect. 2009;58:425–32.
Wieckowska A, Zein N, Yerian L, et al. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatology. 2006; 44:27–33.
Wieckowska A, McCullough AJ, Feldstein AE. Noninvasive diagnosis and monitoring of nonalcoholic steatohepatitis: present and future. Hepatology. 2007;46:582–9.
Xie L, Yui J, Hatori A, et al. Translocator protein (18 kDa), a potential molecular imaging biomarker for non-invasively distinguishing non-alcoholic fatty liver disease. J Hepatol. 2012;57:1076–82.
Yaman H, Cakir E, Akgul EO, et al. Pentraxin 3 as a potential biomarker of acetaminophen-induced liver injury. Exp Toxicol Pathol. 2013;65:147–51.
Yilmaz Y, Dolar E, Ulukaya E, et al. Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from nonalcoholic steatohepatitis. World J Gastroenterol. 2007;13:837–44.
Yoneda M, Mawatari H, Fujita K, et al. High-sensitivity C-reactive protein is an independent clinical feature of nonalcoholic steatohepatitis (NASH) and also of the severity of fibrosis in NASH. J Gastroenterol. 2007;4:573–82.
Yoneda M, Uchiyama T, Kato S, et al. Plasma Pentraxin3 is a novel marker for nonalcoholic steatohepatitis (NASH). BMC Gastroenterol. 2008;8:53.
Yoneda M, Nozaki Y, Endo H, et al. Serum ferritin is a clinical biomarker in Japanese patients with nonalcoholic steatohepatitis (NASH) independent of HFE gene mutation. Dig Dis Sci. 2010;55:808–14.
Younossi ZM, Jarrar M, Nugent C, et al. A novel diagnostic biomarker panel for obesity-related nonalcoholic steatohepatitis (NASH). Obes Surg. 2008;18:1430–7.
Zanetti M, Bosutti A, Ferreira C, et al. Circulating pentraxin 3 levels are higher in metabolic syndrome with subclinical atherosclerosis: evidence for association with atherogenic lipid profile. Clin Exp Med. 2009;9:243–8.

Alpha-Fetoprotein as a Biomarker in Hepatocellular Carcinoma: Focus on Its Role in Composition of Tumor Staging Systems and Monitoring of Treatment Response

30

Stephen L. Chan, Anthony W. H. Chan, and Simon C. H. Yu

Contents

Abstract

Alpha-fetoprotein (AFP) remains the most commonly used biomarker during the management of hepatocellular carcinoma (HCC). AFP is a glycoprotein that is elevated in the serum of approximately 70% of patients with HCC. Over the past five decades, AFP has been extensively studied about its role in diagnosis and surveillance of HCC. In this book chapter, the focus is put on the role of serum

S.L. Chan (✉)
State Key Laboratory of Oncology in South China, Department of Clinical Oncology, Chinese University of Hong Kong, Shatin, NT, Hong Kong
e-mail: chanlam_stephen@cuhk.edu.hk

A.W.H. Chan (✉)
Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Shatin, NT, Hong Kong
e-mail: awh_chan@cuhk.edu.hk

S.C.H. Yu (✉)
Department of Imaging and Interventional Radiology, The Chinese University of Hong Kong, Shatin, NT, Hong Kong
e-mail: simonyu@cuhk.edu.hk

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_41

AFP in the composition of tumor staging systems and monitoring of treatment response for clinical uses in HCC.

Keywords
Liver cancer • Marker • AFP • Hepatitis • Staging systems • Monitoring

List of Abbreviations

AASLD	American Association for the Study of Liver Diseases
AFP	Alpha-fetoprotein
ALBI	Albumin-bilirubin
AUC	Area under curve
CLIP	Cancer of the Liver Italian Program
CUPI	Chinese University Prognostic Index
DCP	Des-carboxy prothrombin
EASL	European Association for the Study of the Liver
GRETCH	Groupe d'Etude et de Traitement du Carcinome Hepatocellulaire
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
MELD	Model for end-stage liver disease
MESIAH	Model to estimate survival in ambulatory HCC patients
PIVKA-II	Prothrombin induced by vitamin K absence-II
TACE	Transarterial chemoembolization
ULN	Upper limit of normal

Key Facts of AFP in the Composition of Staging Systems

- Among patients with HCC, a higher level of serum AFP is predictive of worse clinical outcome.
- A number of tumor staging systems for HCC have incorporated serum AFP value in the systems to improve the prognostication.

Key Facts of AFP in Monitoring of Treatment Response

- A falling trend of serum AFP after treatment is correlated with radiological response and better survival outcomes in HCC.

Definitions of Words and Terms

Diagnosis	Process of determining the underlying disease that explains the patients' symptoms and signs.
Prognosis	Prediction of the severity and likely outcome of a patient's current illness. It typically refers to survival in patients with cancers.
Cancer Screening	A process of testing apparently healthy people for the cancer with an aim to detect cancer at early stage.
Specificity	A statistical measure of performance of a binary classification test, which measures the proportion of true positives among populations who have the condition, also known as the true positive rate.
Sensitivity	A statistical measure of performance of a binary classification test, which measures the proportion of true negatives among populations who do not have the condition, also known as the true negative rate.

Introduction

Alpha-fetoprotein (AFP) is a glycoprotein with a molecular weight of 72 kDa. AFP is primarily produced by the fetal liver with the highest serum level in the fetus, but the level decreases rapidly after birth. The key biological function of AFP appears to be involved in the regulation of fatty acids in fetal liver and proliferating liver cells. The association between AFP and hepatocellular carcinoma (HCC) could be traced back to the 1960s when scientists detected AFP in the circulation of animal models with HCC (Abelev et al. 1963; Stanislawski-Birencwajg et al. 1966; Tatarinov 1979). Afterward, a number of clinical investigators have demonstrated the association of serum AFP and HCC in patients, leading to robust studies to explore the clinical role of measuring serum AFP and establish the use of AFP in clinical setting. Nowadays, it is known that AFP is secreted in approximately 70% of HCCs and the incidence of AFP elevation is higher in endemic regions including Asia and Africa (Purves et al. 1970). Because of low cost and the widespread availability of AFP assays, serum AFP remains one of the mostly commonly used biomarkers during the management of HCC. The diagnostic and surveillance of AFP in HCC has already been reviewed (Kashkoush et al. 2016). In this chapter, two areas of clinical uses of serum AFP, namely, its role in tumor staging system and in monitoring of treatment responses, will be discussed.

Numerous studies have shown that serum AFP value is a modest prognostic marker (Hakeem et al. 2012; Park and Park 2013; Chan et al. 2009a). The mechanism for the prognostic role of AFP in HCC remains obscure. It is generally viewed

that the serum marker is reflective of tumor burden and proliferative activity of HCC (Li et al. 2001, 2002b; Mizejewski 2002; Wang and Xie 1999; Dudich et al. 1998). The prognostic role of serum AFP is not influenced by the treatment modality; higher serum AFP remains a prognostic factor in patients undergoing surgery, liver transplantation, locoablation, transarterial chemoembolization, radiotherapy, or best supportive care (Ikeda et al. 1993; Kumada et al. 1997; Xu et al. 2009; Tateishi et al. 2006; Farinati et al. 2006). Patients with very AFP values, of more than 10,000 ng/ml, were found to have much worse prognosis with a 3-year survival rate of 40%, while those with moderately high AFP, 200–10,000 ng/ml, have a survival rate close to 70% (Ikai et al. 2004). There is no consensus of the cutoff value for serum AFP to be applies as prognostic marker. However, it appears that a higher cutoff value of AFP (600–1,000 ng/ml) is required for prognostication of HCC, as compared to the diagnostic cutoff values (Chan et al. 2009; Farinati et al. 2006).

Cutoff Value AFP for Prognostication in Chinese Patients with HCC

In our cohort of 1,868 patients with HCC, elevation of serum AFP was associated with poorer prognosis (unpublished data). By using the cutoff values of 20 and 400 ng/ml, patients with AFP more than 400 ng/ml had the worst outcome (1- and 5-year overall survival of 41.2% and 19.2%) compared to those less than 20 ng/ml (1- and 5-year overall survival of 77.9% and 48.0%) and >20–400 ng/ml (1- and 5-year overall survival of 53.7% and 35.7%) (Fig. 1a). Among patients (n = 1,123) undergoing palliative treatment (including transarterial therapy, systemic therapy, and best supportive care), serum AFP was a prognostic factor to predict overall survival (Fig. 1b). Among patients (n = 745) receiving curative treatment (surgery and local ablation), serum AFP predicted overall and disease-free survival (Fig. 1c, d). High serum AFP (>400 ng/ml) was associated with aggressive tumor characteristics (larger tumors, multifocal tumors, vascular invasion, and extrahepatic spread) and poorer liver function [higher Child-Pugh grade and higher albumin-bilirubin (ALBI) grade] (Table 1).

Role of AFP in the Composition of Tumor Staging Systems for HCC

Serum AFP alone or in combination with other biomarkers or tumor characteristics is useful in predicting clinical outcome of HCC patients. There are over 15 different staging systems for HCC (Berhane et al. 2016; Chan et al. 2016; Liu et al. 2016). Some of these staging systems incorporate serum AFP as one of the parameters. In 1998, the Cancer of the Liver Italian Program (CLIP) system was derived from a retrospective cohort of 435 HCC patients from 16 Italian hospitals and assess four parameters: tumor morphology, portal vein thrombosis, Child-Pugh grade, and serum AFP (400 ng/ml as the cutoff) (Table 2; The Cancer of the Liver Italian

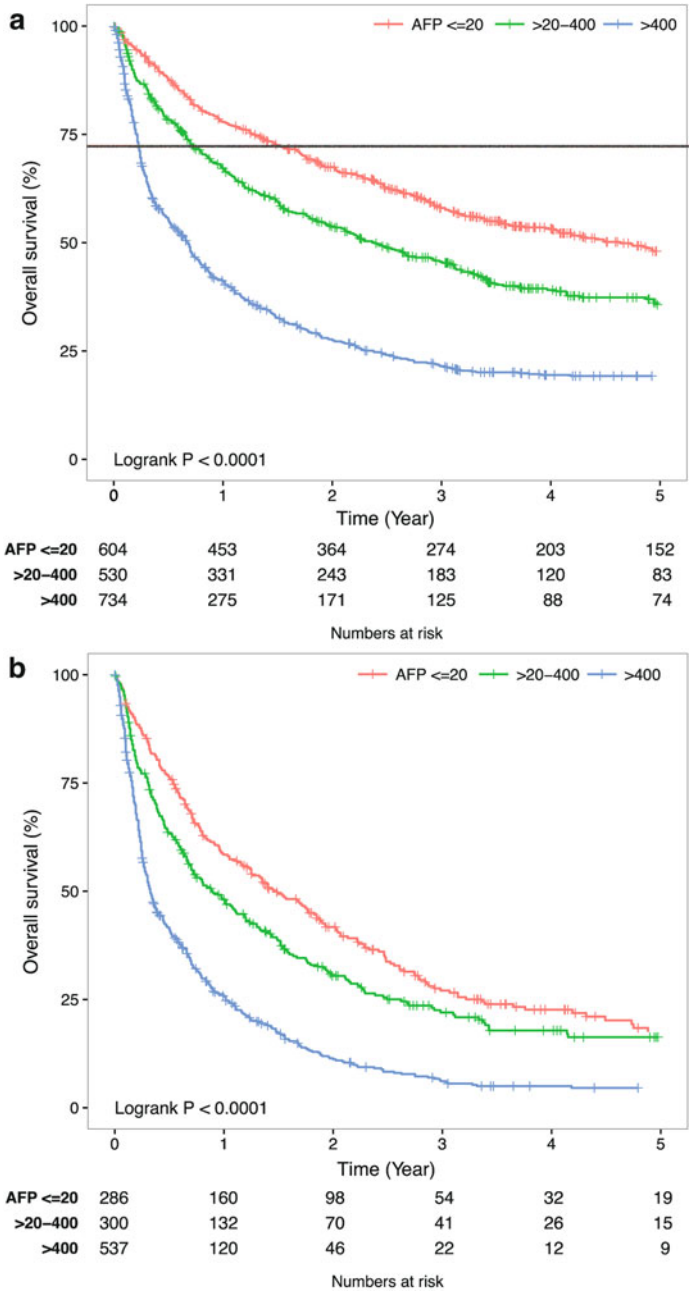

Fig. 1 (continued)

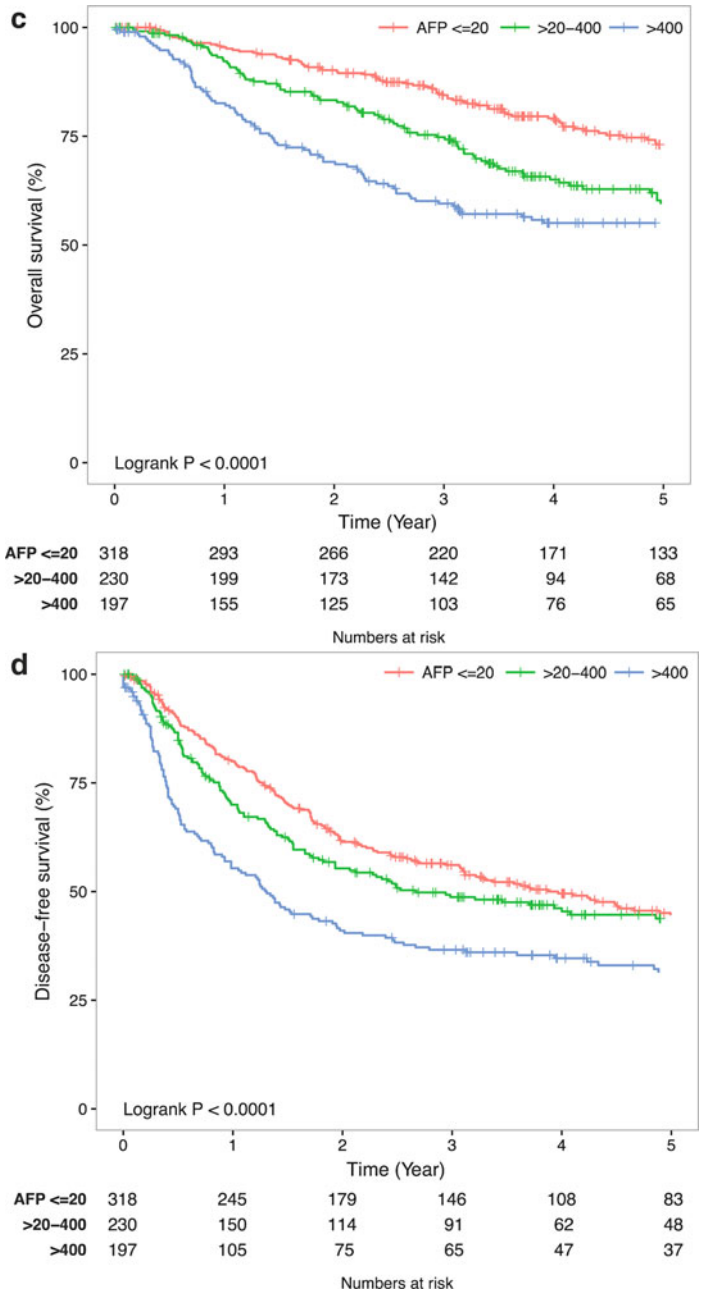

Fig. 1 Kaplan-Meier survival plots evaluating (**a**) overall survival among 1,868 HCC patients, (**b**) overall survival among 1,123 HCC patients undergoing palliative treatment, (**c**) overall survival, and (**d**) disease-free survival among 745 HCC patients receiving curative treatment

Table 1 The mathematical model of the GALAD (Johnson et al. 2014)

Z	=	$-10.08 + 0.09 \times \text{age} + 1.67 \times \text{sex}$ (1, male; 0, female) $+ 2.34 \times \log_{10}\text{AFP} + 0.04 \times \text{AFP-L3} + 1.33 \times \log_{10}\text{DCP}$
Probability of HCC	=	$e^{Z} \div (1 + e^{Z})$

Table 2 The CLIP system (The Cancer of the Liver Italian Program (CLIP) investigators 1998)

	Score		
Parameter	0	1	2
Tumor morphology	Uninodular and extension ≤50%	Multinodular and extension ≤50%	Massive or extension >50%
Portal vein thrombosis	No	Yes	–
Serum AFP (ng/ml)	<400	≥400	–
Child-Pugh grade	A	B	C

Table 3 The GRETCH system (Chevret et al. 1999)

	Score			
Parameter	0	1	2	3
Karnofsky index	≥80	–	–	<80
Portal vein thrombosis	No	Yes		
Serum AFP (ng/ml)	<35		≥35	
Serum bilirubin (μmol/l)	<50			≥50
Serum alkaline phosphatase (ULN)	<2		≥2	

ULN upper limit of normal

Program (CLIP) investigators 1998). In 1999, the Groupe d'Etude et de Traitement du Carcinome Hepatocellulaire (GRETCH) system was developed from a retrospective cohort of 761 patients from 24 Western medical centers and incorporates serum AFP (35 ng/ml as the cutoff), performance status (Karnofsky index), portal vein thrombosis, serum alkaline phosphatase, and total bilirubin (Table 3; Chevret et al. 1999). In 2002, the Chinese University Prognostic Index (CUPI) system was derived from a retrospective cohort of 926 Chinese HCC patients from our center and evaluates serum AFP (500 ng/ml as the cutoff) together with tumor-node-metastasis stage, patient's symptom, ascites, serum alkaline phosphatase, and total bilirubin (Table 4; Leung et al. 2002). In 2006, Toyoda et al. developed BALAD score entirely based on serum markers from a cohort of 2,599 HCC patients from five Japanese hospitals. The BALAD score is based on bilirubin, albumin, AFP (400 ng/ml as the cutoff), AFP-L3 (15% as the cutoff), and DCP (Table 5; Toyoda et al. 2006). In 2012, the model to estimate survival in ambulatory HCC patients (MESIAH) was derived from 477 patients in a single center and incorporates serum

Table 4 The CUPI system (Leung et al. 2002)

Parameter		Score
AJCC 5th TNM stage	I–II	−3
	III	−1
	IV	0
Asymptomatic		−4
Ascites		3
Serum AFP (ng/ml)	≥500	2
Serum bilirubin (μmol/l)	<34	0
	34–51	3
	>51	4
Serum alkaline phosphatase (IU/l)	≥200	3
Risk group		Score
Low		≤1
Intermediate		2–7
High		≥8

Table 5 The BALAD score (Toyoda et al. 2006)

Parameters	Bilirubin-albumin score			
	0	1	2	
Serum albumin (g/l)	>35	28–35	<28	
Serum bilirubin (μmol/l)	<17	17–34	>34	
Parameters	BALAD score			
	0	1	2	3
Bilirubin-albumin score	0–1	2–3	4	–
No. elevated tumor markers	0	1	2	3

AFP (as a continuous variable), age, number of tumor nodules, size of the largest nodule, vascular invasion, metastasis, serum albumin, and the model for end-stage liver disease (MELD) (Yang et al. 2012). In 2014, a newer BALAD-2 model was developed on the same five variables in a continuous rather than a categorical manner (Fox et al. 2014). All these AFP-based prognostic systems and models have been validated internally by prospective cohorts and externally by other groups to provide prognostic information for HCC patients (Berhane et al. 2016; Li et al. 2016; Yang et al. 2012; The Cancer of the Liver Italian Program (CLIP) Investigators 2000; Chan et al. 2011, 2014; Hui et al. 2015). In summary, serum AFP is a prognostic marker for HCC, and its incorporation in the staging systems improves the performance of these systems.

Applicability of AFP in the Monitoring Treatment Response of HCC

The level of serum AFP is shown to be associated with larger tumor size and increased number of tumor in HCC suggesting that serum AFP level is reflective of tumor burden in the body. But whether the serial change of AFP level in serum is

Table 6 Summary of key studies on the performance of serum AFP alone in the surveillance of HCC

Author (year)	N	Incidence of HCC	%HBV/ HCV/alcohol	Cutoff (ng/ml)	Sensitivity (%)	Specificity (%)
Pateron et al. 1994	118	5.8%/year	4.2/0/69.5	100	21	93
Sherman et al.1995	1,069	0.47%/year	100/0/0	20	64.3	91.4
Bolondi et al. 2001	313	4.1% (year)	17.3/64.2/0	20	41	82

HBV hepatitis B virus, *HCC* hepatocelluar carcinoma, *HCV* hepatitis C virus

continuously reflective, the variation in tumor burden requires clinical studies for confirmation. First piece of evidences come from studies, as reported by McIntire et al., in 1972 and 1979 reporting on the natural history of the trend of serum AFP in untreated HCC populations (McIntire et al. 1972, 1976). In summary, it was found that among patient with elevation of serum AFP at baseline, the level would remain static or increasing during progression of HCC, while for HCC patients without detectable AFP in serum, the AFP level would increase at the time of progression. Despite criticisms on small sample size and old assays on AFP, these studies indicate that spontaneous drop of AFP value is highly unlikely during the natural progression of HCC without treatment. Multiple retrospective case series have worked on the clinical value of serum AFP on monitoring of treatment response. In general, for patients undergoing surgical resection of HCC, the serum AFP will decrease remarkably after the surgery and rise at the time of recurrent disease. For patients who receive less effective treatment, usually of palliative intent, such as TACE or cytotoxic chemotherapy, reduction of serum AFP is less frequently observed, but in occasional case of reduction, it is also associated with prolonged overall survival suggestive of treatment response (Table 6).

In 2005, there has been study based on prospective clinical trial published in the Journal of Clinical Oncology (Chan et al. 2009). The study has reviewed the trend of serum AFP in a phase III clinical trials comparing two different chemotherapy regimens. In the study, a wide range of response (10–50%) in AFP value has been studied. It was found that if patients could have at least 20% or more in reduction of serum AFP after 2–3 cycles of treatment (~6–9 weeks), there would be more than doubling of overall survival from 5.6 months to 13.5 months ($p < 0.0001$). It was also shown that the achievement of AFP response was associated with radiologic response. Following this study, a number of similar studies have adopted 20% reduction in serum AFP value in defining serological response in AFP for patients with HCC undergoing different modalities of treatment such as locoablation or sorafenib treatment. For examples, Riaz et al. showed that AFP response, defined as 50% decrease from baseline, after TACE or radioembolization, is associated with higher WHO response (AFP responder, 53%, vs. nonresponder, 24%; $p = 0.002$) and better overall survival (AFP responder, 5.5 months, vs. nonresponders, 2.7 months; hazard ratio = 2.7) (Riaz et al. 2009). Yau et al. also demonstrated

that the achievement of AFP response, defined as >20% reduction after 6 weeks of sorafenib, is associated with better progression-free survival and a trend of better overall survival (Yau et al. 2011). All of these studies consistently demonstrated that AFP response is a surrogate marker for response to anticancer treatment in patients with HCC. This is clinically important because HCC does not frequently shrink in size during TACE or sorafenib treatment. AFP response could assist clinicians in decision-making and identification of responders during drug testing in clinical trials. In fact, an increasing number of clinical trials on HCC incorporate AFP response as the secondary endpoints to determine the efficacy of treatment.

There are three caveats when using serial trend of AFP response for monitoring of treatment response. First, there may be false reduction of serum AFP value if the patient has low range of AFP <200 ng/ml, likely due to lower specificity of AFP for HCC at lower value (Chan et al. 2009). Therefore, clinicians should be cautious in the interpretation of AFP response when the baseline AFP value is lower than 200 ng/ml. Second, patients could have a falsely elevated serum AFP at baseline due to chronic active hepatitis. Any falling AFP value after treatment of cancer and/or hepatitis may be mistakenly considered AFP response. To avoid this, clinicians always interpret the hepatitis by clinical or liver enzymes at baseline as well as interpreting AFP response together with radiological imaging during treatment. Thirdly, so far the clinical meaning of AFP response has been validated in cytotoxic chemotherapy, TACE, and sorafenib treatment. Whether AFP response carries similar meaning in some novel treatment with different mechanisms, such as checkpoint immunotherapeutics, is unclear.

Conclusions

AFP is a useful biomarker in the management of HCC. In addition to diagnosis and surveillance of HCC, the prognostic role of serum AFP can be reflected by its incorporation in a number of existing staging systems for HCC. Serial measurement of serum AFP is helpful in gauging the treatment response during nonsurgical treatment. This will help provide supplemental information when radiological response is not frequently observed after the treatment. The caveats about the clinical use of serum AFP is due to its suboptimal sensitivity. At the same time, despite the relatively high specificity, elevation of AFP is occasionally observed in other cancer types and conditions. Clinicians should always correlate with clinical situation of individual patients when interpreting the serum AFP value in the clinics.

Summary Points

Role in the Composition of Tumor Staging Systems

- Serum AFP level is an independent prognostic marker in HCC, regardless of the disease stage and treatment modality.

- Serum AFP is included in a number of staging systems for HCC such as the BALAD score or the CLIP score, the CUPI system, and the GRETCH score to improve the risk stratification of outcomes of HCC.

Role in the Monitoring of Treatment Response

- For patients who undergo surgical treatment, the level of AFP will typically decrease remarkably and rise at the time of recurrence.
- The reduction in serum AFP after nonsurgical treatment is generally predictive of treatment response with improvement in survival. This applies to TACE, radioembolization, cytotoxic chemotherapy, and sorafenib. Whether AFP is useful in monitoring of treatment response of immune-checkpoint therapy is unknown.

References

Abelev GI, Perova SD, Khramkova NI, Postnikova ZA, Irlin IS. Production of embryonal alpha-globulin by transplantable mouse hepatomas. Transplantation. 1963;1:174–80.

Bolondi L, Sofia S, Siringo S, Gaiani S, Casali A, Zironi G, et al. Surveillance programme of cirrhotic patients for early diagnosis and treatment of hepatocellular carcinoma: a cost effectiveness analysis. Gut. 2001;48:251–9.

Berhane S, Toyoda H, Tada T, Kumada T, Kagebayashi C, Satomura S, et al. Role of the GALAD and BALAD-2 serologic models in diagnosis of hepatocellular carcinoma and prediction of survival in patients. Clin Gastroenterol Hepatol. 2016;14:875–86.

Chan SL, Chan AT, Yeo W. Role of alpha-fetoprotein in hepatocellular carcinoma: prognostication, treatment monitoring or both? Future Oncol. 2009a;5:889–99.

Chan SL, Mo FK, Johnson PJ, Hui EP, Ma BB, Ho WM, et al. New utility of an old marker: serial alpha-fetoprotein measurement in predicting radiologic response and survival of patients with hepatocellular carcinoma undergoing systemic chemotherapy. J Clin Oncol. 2009b;27:446–52.

Chan SL, Mo FK, Johnson PJ, Liem GS, Chan TC, Poon MC, et al. Prospective validation of the Chinese University Prognostic Index and comparison with other staging systems for hepatocellular carcinoma in an Asian population. J Gastroenterol Hepatol. 2011;26:340–7.

Chan SL, Johnson PJ, Mo F, Berhane S, Teng M, Chan AW, et al. International validation of the Chinese university prognostic index for staging of hepatocellular carcinoma: a joint United Kingdom and Hong Kong study. Chin J Cancer. 2014;33:481–91.

Chan AW, Chong CC, Mo FK, Wong J, Yeo W, Johnson PJ, et al. Applicability of Albumin-Bilirubin-based Japan Integrated Staging (ALBI-T) score in hepatitis B-associated hepatocellular carcinoma. J Gastroenterol Hepatol. 2016. doi:10.1111/jgh.13339.

Chevret S, Trinchet JC, Mathieu D, Rached AA, Beaugrand M, Chastang C. A new prognostic classification for predicting survival in patients with hepatocellular carcinoma. Groupe d'Etude et de Traitement du Carcinome Hepatocellulaire. J Hepatol. 1999;31:133–41.

Dudich E, Semenkova L, Gorbatova E, Dudich I, Khromykh L, Tatulov E, et al. Growth-regulative activity of human alpha-fetoprotein for different types of tumor and normal cells. Tumour Biol. 1998;19:30–40.

Farinati F, Marino D, De Giorgio M, Baldan A, Cantarini M, Cursaro C, et al. Diagnostic and prognostic role of alpha-fetoprotein in hepatocellular carcinoma: both or neither? Am J Gastroenterol. 2006;101:524–32.

Fox R, Berhane S, Teng M, Cox T, Tada T, Toyoda H, et al. Biomarker-based prognosis in hepatocellular carcinoma: validation and extension of the BALAD model. Br J Cancer. 2014;110:2090–8.

Hakeem AR, Young RS, Marangoni G, Lodge JP, Prasad KR. Systematic review: the prognostic role of alpha-fetoprotein following liver transplantation for hepatocellular carcinoma. Aliment Pharmacol Ther. 2012;35:987–99.

Hui EP, Ma BB, Chan KC, Chan CM, Wong CS, To KF, et al. Clinical utility of plasma Epstein-Barr virus DNA and ERCC1 single nucleotide polymorphism in nasopharyngeal carcinoma. Cancer. 2015;121:2720–9.

Ikai I, Arii S, Kojiro M, Ichida T, Makuuchi M, Matsuyama Y, et al. Reevaluation of prognostic factors for survival after liver resection in patients with hepatocellular carcinoma in a Japanese nationwide survey. Cancer. 2004;101:796–802.

Ikeda K, Saitoh S, Koida I, Arase Y, Tsubota A, Chayama K, et al. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. Hepatology. 1993;18:47–53.

Johnson PJ, Pirrie SJ, Cox TF, Berhane S, Teng M, Palmer D, et al. The detection of hepatocellular carcinoma using a prospectively developed and validated model based on serological biomarkers. Cancer Epidemiol Biomarkers Prev. 2014;23:144–53.

Kashkoush S, Saleh S, Elmoghazy W. Serum alpha-fetoprotein as a biomarker in liver transplantation. In: Biomarkers in disease: methods, discoveries and applications. 2016, Springer Netherlands. in press.

Kumada T, Nakano S, Takeda I, Sugiyama K, Osada T, Kiriyama S, et al. Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. Hepatology. 1997;25:87–92.

Leung TW, Tang AM, Zee B, Lau WY, Lai PB, Leung KL, et al. Construction of the Chinese University Prognostic Index for hepatocellular carcinoma and comparison with the TNM staging system, the Okuda staging system, and the Cancer of the Liver Italian Program staging system: a study based on 926 patients. Cancer. 2002;94:1760–9.

Li Y, Tang ZY, Ye SL, Liu YK, Chen J, Xue Q, et al. Establishment of cell clones with different metastatic potential from the metastatic hepatocellular carcinoma cell line MHCC97. World J Gastroenterol. 2001;7:630–6.

Li MS, Li PF, He SP, Du GG, Li G. The promoting molecular mechanism of alpha-fetoprotein on the growth of human hepatoma Bel7402 cell line. World J Gastroenterol. 2002a;8:469–75.

Li MS, Li PF, Yang FY, He SP, Du GG, Li G. The intracellular mechanism of alpha-fetoprotein promoting the proliferation of NIH 3T3 cells. Cell Res. 2002b;12:151–6.

Li R, Yang D, Tang CL, Cai P, Ma KS, Ding SY, et al. Combined hepatocellular carcinoma and cholangiocarcinoma (biphenotypic) tumors: clinical characteristics, imaging features of contrast-enhanced ultrasound and computed tomography. BMC Cancer. 2016;16:158.

Liu PH, Hsu CY, Hsia CY, Lee YH, Su CW, Huang YH, et al. Prognosis of hepatocellular carcinoma: assessment of eleven staging systems. J Hepatol. 2016;64:601–8.

McIntire KR, Vogel CL, Princler GL, Patel IR. Serum alpha-fetoprotein as a biochemical marker for hepatocellular carcinoma. Cancer Res. 1972;32:1941–6.

McIntire KR, Vogel CL, Primack A, Waldmann TA, Kyalwazi SK. Effect of surgical and chemotherapeutic treatment on alpha-fetoprotein levels in patients with hepatocellular carcinoma. Cancer. 1976;37:677–83.

Mizejewski GJ. Biological role of alpha-fetoprotein in cancer: prospects for anticancer therapy. Expert Rev Anticancer Ther. 2002;2:709–35.

Park H, Park JY. Clinical significance of AFP and PIVKA-II responses for monitoring treatment outcomes and predicting prognosis in patients with hepatocellular carcinoma. Biomed Res Int. 2013;2013:310427.

Pateron D, Ganne N, Trinchet JC, Aurousseau MH, Mal F, Meicler C, et al. Prospective study of screening for hepatocellular carcinoma in Caucasian patients with cirrhosis. J Hepatol. 1994;20:65–71.

Purves LR, Bersohn I, Geddes EW. Serum alpha-feto-protein and primary cancer of the liver in man. Cancer. 1970;25:1261–70.

Riaz A, Ryu RK, Kulik LM, Mulcahy MF, Lewandowski RJ, Minocha J, et al. Alpha-fetoprotein response after locoregional therapy for hepatocellular carcinoma: oncologic marker of radiologic response, progression, and survival. J Clin Oncol. 2009;27:5734–42.

Sherman M, Peltekian KM, Lee C. Screening for hepatocellular carcinoma in chronic carriers of hepatitis B virus: incidence and prevalence of hepatocellular carcinoma in a North American urban population. Hepatology. 1995;22:432–8.

Stanislawski-Birencwajg M, Frayssinet C, Grabar P. Embryonic antigens in liver tumors in rats. Arch Immunol Ther Exp (Warsz). 1966;14:730–6.

Tatarinov YS. Alpha-Fetoprotein in the laboratory testing for cancer. Gan. 1979;70:133–9.

Tateishi R, Shiina S, Yoshida H, Teratani T, Obi S, Yamashiki N, et al. Prediction of recurrence of hepatocellular carcinoma after curative ablation using three tumor markers. Hepatology. 2006;44:1518–27.

The Cancer of the Liver Italian Program (CLIP) investigators. A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients. Hepatology. 1998;28:751–5.

The Cancer of the Liver Italian Program (CLIP) Investigators. Prospective validation of the CLIP score: a new prognostic system for patients with cirrhosis and hepatocellular carcinoma. Hepatology. 2000;31:840–5.

Toyoda H, Kumada T, Osaki Y, Oka H, Urano F, Kudo M, et al. Staging hepatocellular carcinoma by a novel scoring system (BALAD score) based on serum markers. Clin Gastroenterol Hepatol. 2006;4:1528–36.

Wang XW, Xie H. Alpha-fetoprotein enhances the proliferation of human hepatoma cells in vitro. Life Sci. 1999;64:17–23.

Xu X, Ke QH, Shao ZX, Wu J, Chen J, Zhou L, et al. The value of serum alpha-fetoprotein in predicting tumor recurrence after liver transplantation for hepatocellular carcinoma. Dig Dis Sci. 2009;54:385–8.

Yang JD, Kim WR, Park KW, Chaiteerakij R, Kim B, Sanderson SO, et al. Model to estimate survival in ambulatory patients with hepatocellular carcinoma. Hepatology. 2012;56:614–21.

Yau T, Yao TJ, Chan P, Wong H, Pang R, Fan ST, et al. The significance of early alpha-fetoprotein level changes in predicting clinical and survival benefits in advanced hepatocellular carcinoma patients receiving sorafenib. Oncologist. 2011;16:1270–9.

Part III

Genetic, Histological, Physical, and Imaging Methods

Genetic Biomarkers of Paracetamol (Acetaminophen)-Induced Acute Liver Failure

31

Michael H. Court

Contents

Abstract

Paracetamol (APAP, acetaminophen), one of the most widely used analgesic and antipyretic drugs, is also the single most common cause of acute liver failure (ALF) in many countries, including Scotland, the USA, Sweden, Australia, and Denmark, among others. Based on United States Acute Liver Failure Study Group (ALFSG) data, about half of patients with APAP-induced ALF had consumed a (single time-point) dose that exceeded the recommended maximum daily limit with the intention of self-harm. However, the remaining ALF patients had consumed APAP for therapeutic purposes over a more prolonged period (days to weeks) without the intention of self-harm. Gene sequence variants that impact the risk, severity of symptoms, or outcome for APAP-induced ALF may

M.H. Court (✉)
Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA, USA
e-mail: michael.court@vetmed.wsu.edu

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_27

be used as biomarkers to identify patients at high risk of developing ALF from therapeutic use of APAP who should be advised to avoid or minimize excessive APAP use, determine the need for *N*-acetylcysteine treatment following APAP overdose, and predict whether aggressive symptomatic treatments such as liver transplant are needed. Several genetic variants associated with risk, symptoms, or outcome of APAP-induced ALF have been identified in candidate genes, including *UGT1A*, *CD44*, *CYP3A5*, *GST-P1*, *GST-T1*, *KRT8*, and *TLA*. However, for some genes the associations were dependent on whether the APAP overdose was acute and intentional (*CYP3A5*) or chronic and unintentional (*UGT1A* and *CD44*). Unbiased approaches to genetic variant discovery such as whole-genome association studies have not been reported to date but could reveal novel genes and gene variants for use as biomarkers of APAP-induced ALF.

Keywords

Paracetamol • Acetaminophen • Hepatotoxicity • Acute liver failure • Glucuronidation

List of Abbreviations

ABC	ATP-binding cassette
ALF	Acute liver failure
ALFSG	Acute Liver Failure Study Group
APAP	Paracetamol
CYP	Cytochrome P450
GSS	Glutathione synthetase
GST	Glutathione S-transferase
JNK	c-Jun N-terminal kinase
KRT	Keratin
LTA	Lymphotoxin alpha
NAPQI	*N*-Acetyl-p-benzoquinone imine
PAPS	3′-Phosphoadenosine-5′-phosphosulfate
RIP	Receptor-interacting protein
SULT	Sulfotransferase
TNF	Tumor necrosis factor
UGT	UDP-glucuronosyltransferase

Key Facts of Paracetamol

- Most widely used mild analgesic (pain reliever) and antipyretic (fever reducer) drug in the USA and Europe.
- The International Nonproprietary Name (INN) and British Approved Name (BAN) is paracetamol, while the United States Adopted Name (USAN) and Japanese Accepted Name (JAN) is acetaminophen.
- First synthesized by Harmon Northrop Morse in 1877 while working at Johns Hopkins University.

- Clinical trials conducted by Joseph von Mering and chemists at Bayer in Germany in 1887 showed analgesic and antipyretic effects, but also significant methemoglobinemia in some patients, probably because of a toxic impurity (4-aminophenol), leading to abandonment of further development for nearly 60 years.
- The "rediscovery" and commercial marketing of paracetamol in the 1950s with the trade names Tylenol® in the USA and Panadol® in the UK were driven by the need to develop alternatives to aspirin for long-term use that have less gastrointestinal irritancy and do not inhibit blood clotting (adverse effects of aspirin).
- The molecular mechanism for the analgesic and antipyretic effects is not well understood but may involve effects on cyclooxygenase and/or the endogenous cannabinoid system.

Key Facts of Paracetamol Toxicity

- The therapeutic index (minimum toxic dose divided by maximum therapeutic dose) of paracetamol is relatively narrow (only two- to threefold) compared with other drugs that are available without a prescription.
- Toxicity is primarily manifest as acute liver injury, and signs (nausea, vomiting, sweating, pain) only become evident hours after overdose with death occurring from days to weeks later.
- The molecular mechanism for toxicity involves conversion in the liver to a reactive metabolite that causes cell death.
- First cases of acute liver injury from overdose were reported in 1966.
- Currently, paracetamol overdose is the single most common cause of acute liver failure in the USA, Europe, and many other Western countries.
- *N*-Acetylcysteine administered by intravenous infusion is an effective antidote if given soon after overdose.
- Fasting, preexistent liver injury, or chronic alcohol use may aggravate liver injury.
- Polymorphisms in various genes including *UGT1A*, *CD44*, *CYP3A5*, *GST-P1*, *GST-T1*, *KRT8*, and *TLA* have been associated with altered risk and/or outcome of liver injury from overdose.

Definitions of Words and Terms

Allele	One of a number of different forms (sequences) of a gene; most genes have two possible alleles that may either be the same (homozygous) or different (heterozygous) since they are present on each of the chromosome pairs.

Candidate genes	Genes encoding proteins likely to contain sequence variants that influence the disease or toxicity outcome.
CYP	Cytochrome P450 enzyme that catalyzes oxidation or reduction of a drug.
Genotype	A description of the gene reference and variant alleles that an individual possesses.
Glucuronidation	The addition of a sugar group (glucuronide) to a drug that is mediated by the UDP-glucuronosyltransferase (UGT) enzymes.
Glutathione	A cysteine containing peptide molecule synthesized within many tissues in a reduced state that protects against oxidant injury through conjugation with highly reactive molecules.
GST	Glutathione S-transferase enzyme that catalyzes addition of glutathione to a reactive compound.
N-Acetylcysteine (NAC)	A drug used to treat paracetamol toxicity through replenishment of reduced glutathione stores.
N-Acetyl-p-benzoquinone imine (NAPQI)	A highly reactive product of metabolism of paracetamol by drug-metabolizing enzymes in the liver.
Reference allele (also called wild-type allele)	The first form of the gene that was sequenced and is usually the most common allele found in the major populations that are studied.
SULT	Sulfotransferase enzyme that catalyzes addition of a sulfate to a drug.
UGT	UDP-glucuronosyltransferase enzyme that catalyzes addition of a glucuronide sugar to a drug.
Variant allele (also called mutant allele)	A form of the gene with a different DNA sequence from the reference allele.

Introduction

Paracetamol (INN; *N*-acetyl-*p*-aminophenol, APAP), also known as acetaminophen, is one of the most widely used analgesic and antipyretic drugs (Kaufman et al. 2002). Although considered to be safe and effective when used at the recommended dosages, APAP overdose can result in severe liver injury leading to acute liver failure (ALF) that may require liver transplantation. An ongoing study by the Acute Liver Failure Study Group (ALFSG) in the USA has clearly demonstrated that APAP is the single most common cause of ALF in that country (Fig. 1; Lee 2012). This study also showed that while approximately half of all cases of APAP-

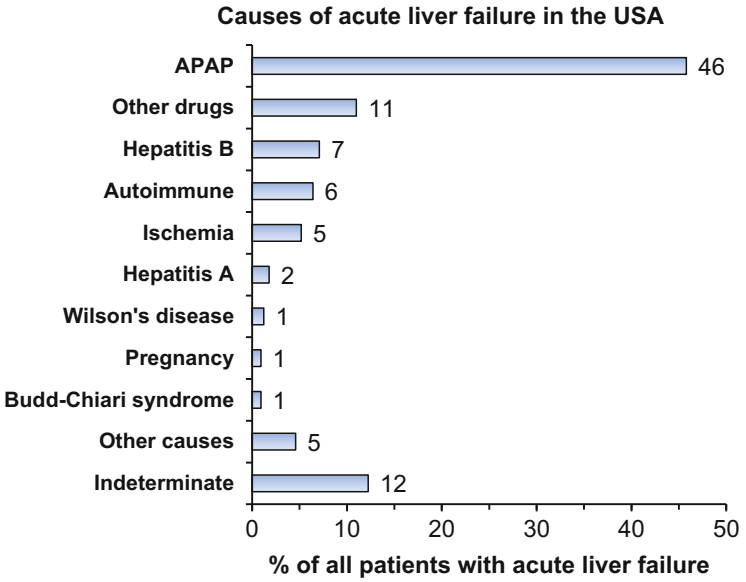

Fig. 1 Causes of acute liver failure (*ALF*) identified in 1,696 patients in the adult registry of the Acute Liver Failure Study Group in the USA from 1998 to 2012. Paracetamol (*APAP*) ingestion is the single most common cause of ALF (Data used to construct the graph were obtained from (Lee 2012))

induced ALF were a consequence of intentional self-harm with a high APAP dose consumed over a short period, the remaining cases were unintentional with multiple doses consumed over days to weeks. Furthermore, the application of a newly developed APAP adduct biomarker assay has revealed that as many as 20% of cases of ALF without an apparent cause (i.e., "indeterminate") are a consequence of unrecognized APAP toxicity (Khandelwal et al. 2011). A United States Center for Disease Control study published in 2011 estimated an annual rate of between 40,000 and 80,000 APAP overdose-related visits to the emergency department and about 34,000 APAP overdose-related hospitalizations per year indicating a significant burden on the healthcare system (Manthripragada et al. 2011). Although the survival rate of APAP-intoxicated patients with appropriate medical treatment is relatively high (66%) compared with other causes of ALF (21–51%), about 9% of cases receive liver transplant at considerable cost.

Studies have also shown that APAP is the leading cause of ALF (36–74% of cases) in other Western countries including Scotland (Bretherick et al. 2011), Sweden (Wei et al. 2007), and Australia (Gow et al. 2004; Fig. 2). Moderate frequencies (11–19%) of APAP-induced ALF were also reported in Denmark, Germany, Canada, and China (Zhao et al. 2013), while low frequencies (2–4%) were reported in Lithuania (Adukauskiene et al. 2008), Spain (Rodriguez Lopez et al. 2012), Hong Kong (Chan 1996), and France (Ostapowicz and Lee 2000). Furthermore, studies in both Argentina (154 ALF cases) (Mendizabal et al. 2014)

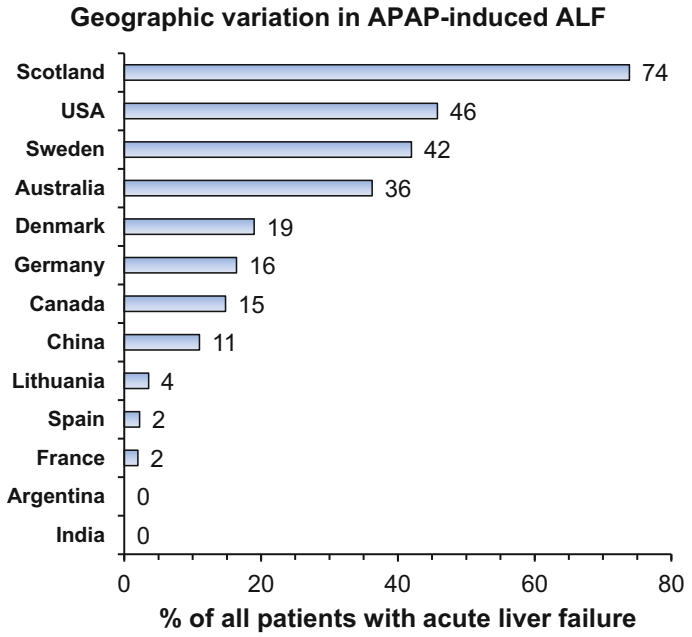

Fig. 2 Geographic variation in the incidence of paracetamol (APAP)-induced acute liver failure (*ALF*). Data are compiled from various studies that were reported from 1996 to 2014 (Chan 1996; Ostapowicz and Lee 2000; Gow et al. 2004; Wei et al. 2007; Adukauskiene et al. 2008; Bretherick et al. 2011; Rodriguez Lopez et al. 2012; Zhao et al. 2013; Mendizabal et al. 2014)

and India (423 ALF cases) (Ostapowicz and Lee 2000) failed to identify any cases of APAP-induced ALF. Various reasons for these large differences between countries have been proposed (Polson and Lee 2007), including the availability and use of alternate methods of self-harm, limitations in patient access to large amounts of APAP, availability of APAP in combination with other drugs (particularly opioids), and differences in the prevalence of alcohol use (see mechanism below). It has also been suggested that these geographic differences may be a consequence of population differences in gene variants that predispose individuals to enhanced or reduced risk of APAP toxicity (Mendizabal et al. 2014).

The purpose of this chapter is to review all published original research studies that have explored the association of genomic variation with APAP-induced ALF in patients with the ultimate goal of using these variants as predictive biomarkers of toxicity risk and outcome. It should be pointed out that the focus here is adult patients since the gene association studies reported to date exclusively involve this demographic group. However, the results from the ongoing multinational Pediatric Acute Liver Failure (PALF) study (Leonis et al. 2013) indicate that APAP is also an important cause of ALF in pediatric patients, and given the additional complication of developmental changes in drug metabolism and toxicity, future studies are needed in this population.

Molecular Mechanisms of APAP-Induced Acute Liver Failure

Accurate interpretation of gene association studies is predicated upon an adequate understanding of the mechanisms underlying APAP-induced liver toxicity. Fortunately, APAP is a prototypical hepatotoxic drug, and there have been a multitude of studies published primarily using animal, tissue, and cellular models and also human subjects (affected patients and volunteers). A search of the PubMed database identified over 1,600 original research papers that date back to 1973 (42 years ago). There have been several recent reviews on this topic (McGill and Jaeschke 2013, 2014; Jaeschke et al. 2014; Lancaster et al. 2015; Mazaleuskaya et al. 2015), and so the most important and relevant findings will be summarized below.

Figure 3 illustrates the main molecular pathways involved in APAP-induced liver injury in the hepatocyte. The essential intoxicant is *N*-acetyl-p-benzoquinone imine (NAPQI), a highly reactive oxidative metabolite of APAP that is formed by hepatic cytochrome P450 (CYP) enzymes. At therapeutic doses (up to 4 g per day) (Gelotte et al. 2007), the majority of drug is metabolized to inactive metabolites by glucuronidation (~60% of dose) and sulfation (~20%) which are primarily excreted in the urine. A small percentage of APAP (~10%) is also oxidized to NAPQI that is normally rapidly detoxified by conjugation with glutathione, which is then further metabolized to thiol derivatives (mainly cysteine and mercapturate conjugates) that are excreted in urine. However, at potentially toxic doses (over 8 g per dose), a lower proportion of drug is sulfated (~10%) probably as a consequence of limited cofactor (3′-phosphoadenosine-5′-sulfate) availability, while a higher proportion of APAP is glucuronidated (~67%) and oxidized to thiol derivatives (~15%) (Prescott 1980). Importantly, the production of NAPQI exceeds the available supply glutathione resulting in significant covalent binding of NAPQI with other molecules in the hepatocyte.

Although the specific molecular targets responsible for NAPQI-induced toxicity have not been yet identified, several lines of evidence indicate that covalent binding of NAPQI with proteins in the mitochondria is essential (Jaeschke et al. 2012, 2014; McGill and Jaeschke 2014). This is thought to cause initial oxidative stress that initiates a signaling cascade leading to activation of c-Jun N-terminal kinase (JNK) via a number of kinases, including mixed lineage kinase 3, apoptosis signal-regulating kinase 1, and receptor-interacting protein (RIP) kinases 1 and 3 (Xie et al. 2014). Activated JNK translocates into the mitochondria causing collapse of the mitochondrial membrane potential, loss of ATP production, and eventually cell death.

There is some controversy as to whether cell death occurs via necrosis or apoptosis or a combination of both mechanisms, which has implications with regard to possible preventatives and treatment (Jaeschke et al. 2012, 2014; McGill and Jaeschke 2014). Although there is DNA laddering suggestive of apoptosis, there is no evidence for involvement of pro-apoptotic caspases. Instead, it has been suggested that DNA laddering results from release of endonuclease G and apoptosis-inducing factor from the mitochondrial intermembrane initially caused by formation of a Bcl2-associated X protein pore in the outer membrane (Jaeschke

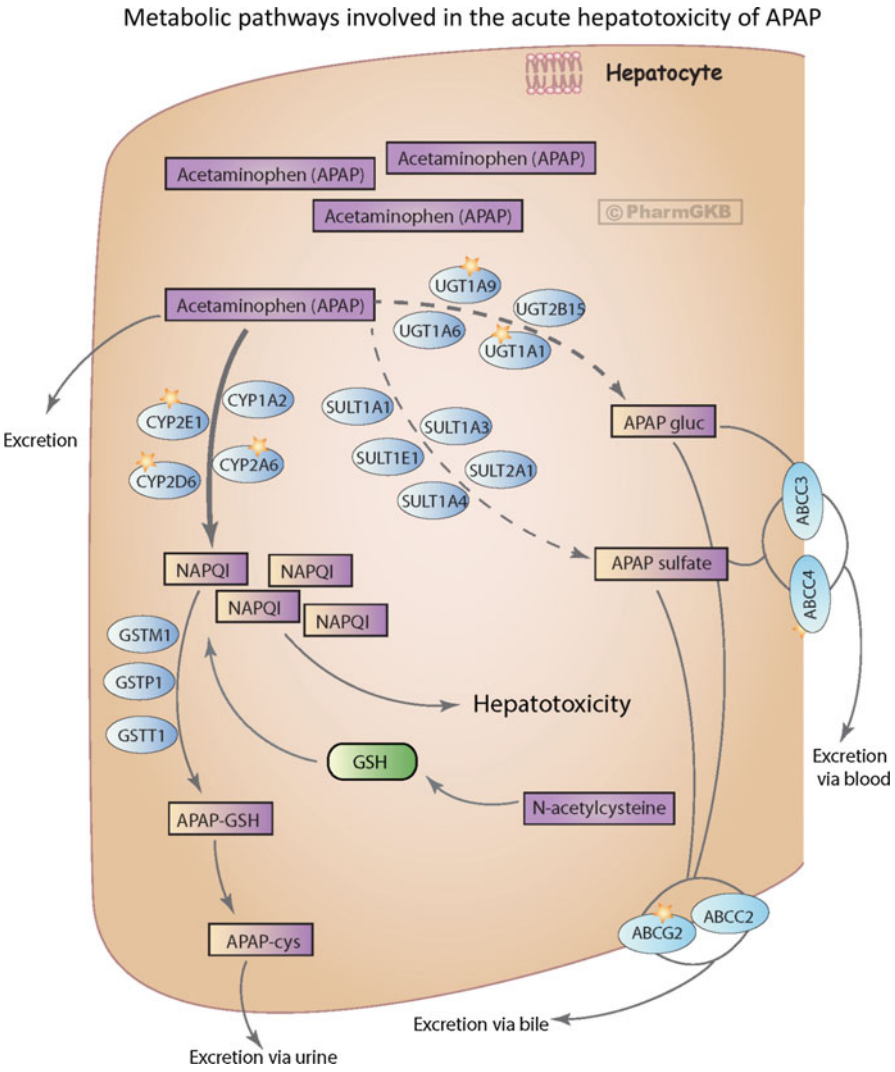

Fig. 3 Metabolic pathways involved in the acute hepatotoxicity of APAP. At therapeutic dose, APAP is primarily metabolized to inactive metabolites including APAP glucuronide (*APAP gluc*) by the UDP-glucuronosyltransferases (*UGTs*) and APAP sulfate (APAP sulfate) by the sulfotransferases (*SULTs*). Unchanged APAP and metabolites are eliminated in urine and bile. A small proportion of drug is also metabolized by cytochrome P450 enzymes to form the reactive metabolite *N*-acetyl-p-benzoquinone imine (*NAPQI*) that is rapidly conjugated by reduced glutathione (*GSH*) either spontaneously or by glutathione S-transferase (*GST*) enzymes. The resulting glutathione adduct is hydrolyzed and excreted in the urine. However, at toxic doses (as depicted here), the capacity to sulfonate and glucuronidate is exceeded, and glutathione stores are depleted, leaving NAPQI available to covalently bind proteins leading to toxicity. Once glutathione stores are depleted, NAPQI is free to covalently bond with *N*-acetylcysteine (*NAC*) that is a precursor of glutathione and is commonly administered to overdose patients with substantial clinical benefit especially if given soon after ingestion has occurred. Figure is from the Pharmacogenomics Knowledge Base website at https://www.pharmgkb.org/pathway/PA166117881 (Reproduced with permission from PharmGKB and Stanford University)

et al. 2014). The ratio of caspase cleaved to intact cytokeratin 18 in the serum has been used as a biomarker of apoptotic versus necrotic cell death in APAP-induced ALF (Possamai et al. 2013). This study suggested that apoptosis may be transiently involved in the early stages of APAP-induced ALF but probably only accounts for about 15% of cell death (McGill and Jaeschke 2014). Furthermore, cleaved caspase-3 or caspase-3 activity (other biomarkers of apoptosis) was not detected in the plasma of APAP hepatotoxicity patients (McGill et al. 2012).

Immune mechanisms including activation of hepatic Kupffer cells, release of cytokines and chemokines, and recruitment of neutrophils and macrophage infiltration follow the initial hepatocyte injury phase and are proposed to be beneficial to effective liver repair and regeneration (Jaeschke et al. 2012).

APAP Hepatotoxicity Candidate Genes

Based on the preceding discussion, candidate genes that could contain genetic variants that affect the risk for developing APAP-induced ALF or might modulate the outcome of the toxicity (such as recovery with medical treatment, liver transplant, or death) include those encoding for proteins involved in determining the amount of NAPQI that is formed (i.e., APAP absorption, distribution, metabolism, and excretion) and those involved in determining or modulating the downstream toxic effects of NAPQI after covalent binding. The following genes described below are not exhaustive but serve to illustrate the core set of genes most likely involved in the pathway leading to APAP hepatotoxicity.

APAP Absorption, Distribution, Metabolism, and Excretion Genes

After dosing, APAP is essentially completely absorbed from the small intestine with about 25% of the dose cleared by first-pass metabolism resulting in a moderately high (~75%) systemic availability (Ameer and Greenblatt 1977). Time to maximal plasma concentration is dependent on the rate of gastric emptying and varies from 40 to 60 min (Dordoni et al. 1973). Radiolabeled drug studies indicate that 90–100% of the drug is eliminated in the urine as parent drug and metabolites within 24 h after dosing (Mitchell et al. 1974). Plasma protein binding of APAP is negligible (<25%) (Bailey and Briggs 2004), while the volume of distribution is about 1 l per kg indicating moderate tissue distribution. Plasma clearance is relatively fast (5 mL/kg/min) with resultant short elimination half-life of 2–4 h. The majority of the drug (~99%) is metabolized to glucuronide, sulfate, and oxidative thiol metabolites (mainly) in the liver before excretion.

Hepatic UDP-glucuronosyltransferase (UGT) enzymes that are involved in APAP glucuronidation include UGT1A1, UGT1A6, UGT1A9, and UGT2B15. Because of their different enzyme kinetic properties, the relative contribution of each of these UGTs to total APAP glucuronidation likely varies according to substrate concentration with UGT1A6 and UGT2B15 being most active at therapeutic APAP concentrations (<100 μM), while UGT1A1 and UGT1A9 are most active at toxic

concentrations (>1 mM) (Mutlib et al. 2006). Of note is that all UGT1A isoforms are encoded by a single gene through differential splicing and there is extensive linkage disequilibrium of variants across the gene (Court et al. 2013). Consequently variants located within or near the UGT1A gene could influence UGT1A enzyme function either through linkage or by affecting the shared exons encoding the C-terminal portion of the enzyme.

UDP-glucuronic acid, the essential glucuronidation cofactor, is synthesized from UDP-glucose by UDP-glucose dehydrogenase. UDP-glucuronic acid concentrations in the liver approximate Km values for APAP glucuronidation and are significantly reduced in rodent models of APAP hepatotoxicity without affecting UDP-glucose concentrations suggesting UDP-glucose dehydrogenase activity could determine APAP detoxification (Hjelle 1986).

Based on recombinant enzyme studies, sulfotransferase (SULT) enzymes that are primarily involved in APAP sulfation at therapeutic concentrations (<100 μM) include SULT1A1, SULT1A3, and SULT1C4 (Yamamoto et al. 2015). SULT1E1 and SULT2A1 may also contribute to activity at high (>1 mM) APAP concentrations. However, the expression of these isoforms differs between tissues and with developmental age (fetal versus adult) (Riches et al. 2009). In adult liver, SULT1A1 is probably the most active form at therapeutic concentrations, while SULT2A1 and possibly SULT1E1 (with somewhat lower abundance than SULT2A1 or SULT1A1) may contribute at toxic concentrations (Yamamoto et al. 2015). SULT1A3 is mainly expressed in the gastrointestinal tract, platelets, brain, and fetal liver (Javitt et al. 2001), while SULT1C4 is mainly expressed in the kidney, ovary, and fetal lung (Sakakibara et al. 1998).

Toxicity may be influenced by the liver concentrations of 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the essential cofactor for sulfation reactions. PAPS is synthesized from ATP and inorganic sulfate by two paralogs of PAPS synthase (PAPSS), PAPSS1 and PAPSS2. PAPSS2 appears to be the main form expressed in adult human liver (http://www.genecards.org/). Maintenance of sufficient sulfate in the liver for PAPS synthesis may also be important for APAP toxicity. Sulfate levels are regulated by the sodium/sulfate symporter (NaS1) encoded by the solute carrier (SLC) transporter 13A1 gene that is responsible for sulfate reabsorption by the kidney (Lee et al. 2006).

Recombinant enzyme studies indicate a major role for CYP3A4 in forming NAPQI in human liver at both therapeutic (50 μM) and toxic (1 mM) APAP concentrations (Patten et al. 1993; Laine et al. 2009). CYP2E1 also showed significant recombinant enzyme activity with additional contributions from CYP1A2, CYP2D6, and CYP2C19 (Laine et al. 2009). These results contrast with studies of human liver microsomes (at therapeutic and toxic concentrations) and drug-drug interaction studies in human subjects (therapeutic doses) that indicate CYP2E1 may be more important than CYP3A4 for NAPQI formation (Thummel et al. 1993; Manyike et al. 2000; Hazai et al. 2002). Since both of these enzymes are highly inducible (by coadministered drugs, alcohol use, and diet) and CYP3A4 shows high interindividual variability in expression, it is likely that the relative importance of each of these enzymes for APAP intoxication could vary within the population.

NAPQI conjugation with glutathione is thought to involve both enzyme-dependent and enzyme-independent mechanisms (Coles et al. 1988). Although GST-P1, GST-T1, and GST-M1 have been shown to catalyze the conjugation of glutathione with NAPQI, there are differences in tissue expression. GST-M1 is abundantly expressed in human liver, while GST-P1 and GST-T1 appear to be expressed at relatively low levels in this tissue (http://www.genecards.org/). Glutathione levels in the liver could also influence toxicity. Glutathione is synthesized in the liver from L-glutamate and L-cysteine in a two-step process. The first rate-limiting step that forms gamma-glutamylcysteine is catalyzed by the heterodimeric glutamate cysteine ligase with a catalytic subunit encoded by the GCLC gene and the regulatory subunit encoded by the GCLM gene. The second step that forms glutathione is catalyzed by glutathione synthetase (GSS).

In addition to detoxification by glutathione, there is some evidence that NAPQI may also undergo reductive metabolism to the parent hydroquinone by NAD(P)H/quinone oxidoreductase 1 with the potential for further metabolites to stable metabolites (Vredenburg et al. 2014).

Membrane transporters are required for elimination of the APAP metabolites. Transport of APAP sulfate and glucuronide into bile involves ATP-binding cassette (ABC) transporters ABCC2 and ABCG2, while APAP glutathione is transported by ABCC2 (Zamek-Gliszczynski et al. 2005, 2006b). However, it should be pointed out that humans excrete relatively little of these metabolites (<3% of dose) into the bile (Siegers et al. 1984). Transport of APAP glucuronide into blood involves ABCC3, while APAP sulfate is transported by ABCC3 and ABCC4 (Manautou et al. 2005; Zamek-Gliszczynski et al. 2006a). It is unclear what transporter mediates basolateral efflux of APAP glutathione conjugates into blood.

Genes encoding transcriptional factors that regulate ADME genes involved in APAP toxicity are also likely candidate genes. Based on mouse knockout studies (Enomoto et al. 2001; Okawa et al. 2006; Liu et al. 2013), the transcription factor Nrf2 (encoded by *NFE2L2*) and the associated oxidant sensor Keap-1 are key effectors of the oxidative stress response and protect against APAP-induced hepatotoxicity through regulating conjugation enzymes (including UGTs, SULTs, and GSTs), transporters, and other antioxidant molecules. Other ADME-regulating transcription factors that may be involved in APAP-induced ALF include pregnane X receptor (PXR, encoded by *NR1I2*) (Cheng et al. 2009), constitutive androstane receptor (CAR, encoded by *NR1I3*) (Zhang et al. 2002), retinoid X receptor alpha (RXR-alpha, encoded by *NR2B1*) (Wu et al. 2004), liver X receptor alpha (LXR-alpha, encoded by *NR1H3*) (Saini et al. 2011), farnesoid X receptor (FXR, encoded by *NR1H4*) (Lee et al. 2010), and peroxisome proliferator-activated receptor alpha (PPAR-alpha, encoded by *NR1C1*) (Chen et al. 2000).

APAP Toxicity Genes

As indicated above, a number of signal transduction pathway molecules (primarily kinases) have been found that are critical to the activation of JNK in APAP

hepatotoxicity, including MLK3, ASK1, and RIP1 and RIP3 (Gunawan et al. 2006; Xie et al. 2014, 2015). Other molecules are likely to be discovered as the toxicity pathway continues to be more fully elucidated. Genes encoding molecules important for progression and repair of hepatic injury are also likely to influence the ultimate outcome but may not substantially affect the risk for developing ALF from APAP.

Nongenetic Factors Affecting APAP-Induced ALF

Nongenetic factors that modify risk for APAP-induced hepatotoxicity and outcome are important to control genetic association studies. Identified risk factors in humans are listed in Table 1. APAP dose and the duration of dosing are likely to influence the likelihood of APAP-induced ALF (Larson et al. 2005). However, a recent study suggests that, at least with intentional acute APAP overdose, outcome (death, transplant, or spontaneous survival) in patients that have already developed ALF may be unrelated to APAP dose suggesting a dose-response plateau effect (Gregory et al. 2010).

APAP ALF Candidate Gene Studies

To date five studies (summarized in Table 2) have been published that evaluated the possible association of variants in 14 different candidate genes with the risk, severity of symptoms, and/or outcome in patients with APAP-induced ALF.

The first study examined the association of tumor necrosis factor (TNF, also known as TNF-alpha or TNFA) and lymphotoxin alpha (LTA, also known as TNF-beta or TNFB) genotypes with risk, symptoms, and outcome in 97 patients with severe APAP hepatotoxicity (Bernal et al. 1998). Variants examined included rs1800629 (G/A) in the *TNF* 5′-flanking region and rs909253 (C/T on reverse strand, G/A on forward strand) in the *LTA* first intron. They found no significant associations of either gene variant with various indices of toxicity severity including median peak international normalized ratio (INR, a blood-clotting measure), blood bilirubin concentration at admission, or median Acute Physiology and Chronic Health Evaluation (APACHE) III score (a clinical measure of disease severity used to predict outcome). There was also no association of either genotype with outcome (spontaneous survival versus died/transplanted). However, there was a weak but statistically significant association ($X^2 = 6.8$, $p = 0.03$) between *TLA* rs909253 C/C genotype and peak encephalopathy severity with a lower genotype frequency of patients in the moderate to severe encephalopathy group (1/46 patients = 2%) compared with the mild/no encephalopathy group (9/51 patients = 18%). The genotype frequency in a matched control population was 11% (12/109 subjects). The authors suggested that both genes may not play a direct role in APAP-induced hepatotoxicity, but *TLA* rs909253 C/C genotype may protect against hepatic encephalopathy mediated by TNF produced by sepsis in ALF patients. In support of this, mouse genetic studies showed that deletion of both the TNF and LTA genes had no effect on

Table 1 Nongenetic factors that have been associated with altered risk of APAP-induced hepatotoxicity in humans

Risk modifier	Effect on risk	Mechanism	Reference
Fasting	Increased	Unclear, possibly reduced hepatic UDP-glucuronic acid and glutathione concentrations	Whitcomb and Block (1994)
Nonalcoholic fatty liver disease	Increased	Unclear, possibly higher CYP2E1 or mitochondrial dysfunction	Michaut et al. (2014)
Chronic liver disease	Increased	Unclear, possibly reduced APAP glucuronidation	Myers et al. (2008)
Chronic alcohol use	Increased	Induction of CYP2E1 (and possibly other CYPs), decreased hepatic glutathione concentrations	Suzuki et al. (2009)
Fibrates	Decreased	Unclear, possibly PPAR-alpha-mediated induction of mitochondrial uncoupling protein 2 (UCP2)	Suzuki et al. (2009)
Nonsteroidal anti-inflammatory drugs, statins, angiotensin-converting enzyme inhibitors, angiotensin receptor II antagonists	Decreased	Unknown	Suzuki et al. (2009)

APAP-induced hepatotoxicity (Boess et al. 1998). Furthermore, serum TNF levels were elevated in ALF patients (Singhal et al. 2009), and TNF inhibition has been proposed as a treatment for hepatic encephalopathy (Butterworth 2015). It is unclear whether the *TLA* rs909253 polymorphism results in a direct effect on TLA expression or through indirect effects on TNF expression since the genes are adjacent to each other, and so there could be significant genetic linkage. In support of the latter, TNF concentrations were lower in the plasma of severely septic patients with the *TLA* rs909253 C/C genotype compared with C/T and T/T genotypes (Stuber et al. 1996). Furthermore, an extended haplotype involving both TNF and LTA genes was a better predictor of an asthma phenotype than genotypes for individual genes (Randolph et al. 2005).

In the second study, the association of polymorphisms in the genes encoding GST-T1, GST-M1, and GST-P1 with risk, prothrombin times (as a predictive marker of survival), and outcome was reported for 104 Danish patients with APAP-induced hepatotoxicity (Buchard et al. 2012). Polymorphisms examined included rs1695 (c.313A > G, p.Ile105Val) in GST-P1 and copy number variations (gene deletion polymorphism) in GST-T1 and GST-M1. Homozygous deletion of the *GST-T1* gene demonstrated a borderline significant association ($P = 0.05$) higher through prothrombin times (better prognosis). Other GST genotypes were not associated with prothrombin time differences. Furthermore, no association with outcome (death or

Table 2 Summary of candidate gene studies that have evaluated genetic associations with APAP-induced hepatotoxicity in humans

Study	Patients	Controls	Genes/variants	Results
(Bernal et al. 1998)	Total of 97 English patients with APAP-induced ALF: 91 with deliberate overdose and 6 with accidental overdose	109 race-/ethnicity-matched subjects for risk assessment	*TNF* rs1800629	No association with risk, symptom severity, or outcome
			TLA rs909253	Weak association of CC genotype with decreased severity of encephalopathy, no association with risk or outcome
(Buchard et al. 2012)	104 Danish patients with APAP-induced ALF	1226 race-/ethnicity-matched subjects for risk assessment	*GST-P1* rs1695	Lower CC genotype (low-activity) frequency ($P = 0.047$) in patients versus matched population (lower risk allele), no association with prothrombin times or outcome
			GST-T1 CNV	Homozygous deletion weakly associated ($P = 0.05$) with higher prothrombin times (better prognosis), no association with outcome, risk association not evaluated
			GST-M1 CNV	No association with prothrombin times or outcome, risk association not evaluated
(Strnad et al. 2010)	Total of 344 patients with ALF: 167 with APAP-induced ALF and 177 with other causes of ALF	583 race-/ethnicity-matched subjects for risk assessment	*KRT8* R341H	Higher variant allele frequency (higher risk) in white patients with ALF (all causes) versus control. No

(*continued*)

Table 2 (continued)

Study	Patients	Controls	Genes/variants	Results
				association with outcome
			KRT8 G434S	Higher variant allele frequency (higher risk) in African American patients with ALF (all causes) versus control. No association with outcome
			KRT8/*KRT18* combined nonconservative coding variants	Borderline association ($P = 0.05$) with survival in multivariate analysis of APAP-induced ALF subjects
(Court et al. 2013)	Total of 260 white American patients with ALF: 79 with unintentional APAP-induced ALF, 78 with intentional APAP-induced ALF, 103 with other causes of ALF	Comparisons made between ALF patients grouped by etiology and to 922 race-/ethnicity-matched subjects for risk assessment	*UGT1A* rs8330	Lower variant allele frequency (lower risk) in white patients with unintentional APAP-induced ALF versus patients with intentional APAP-induced ALF and patients with other causes of ALF. Association with symptom severity or outcome not assessed
(Court et al. 2014)	Total of 260 white American patients with ALF: 79 with unintentional APAP-induced ALF, 78 with intentional APAP-induced ALF, 103 with other causes of ALF	Comparisons made between ALF patients grouped by etiology and to 60 race-/ethnicity-matched subjects for risk assessment	*CYP3A5* rs776746	Higher variant allele frequency (higher risk) in white patients with intentional APAP-induced ALF versus patients with intentional APAP-induced ALF and patients with other causes of ALF.

(*continued*)

Table 2 (continued)

Study	Patients	Controls	Genes/variants	Results
				Association with symptom severity or outcome not assessed
			CD44 rs1467558	Higher A/A genotype frequency (higher risk) in white patients with intentional APAP-induced ALF versus patients with intentional APAP-induced ALF and patients with other causes of ALF. Association with symptom severity or outcome not assessed
			UGT1A1 (TA) n;	No association of allele frequencies with risk. Associations with symptom severity or outcome not assessed
			UGT1A6 rs6759892 rs2070959 rs1105879	
			UGT1A9 rs6714486	
			UGT2B15 rs1902023	
			SULT1A1 rs9282861	
			*CYP2E1*1D*	
			*CYP2E1*1x2*	
			BHMT1 rs3733890	

ALF acute liver failure, *APAP* paracetamol

hepatic encephalopathy) was found for any of the *GST* genotypes evaluated. However, they did find a lower than expected frequency of the *GST-P1* rs1695 homozygous variant (G/G) low-activity genotype of 4.8% in their APAP-intoxicated cohort when compared to the genotype frequency of 11% in the healthy Danish population. The association between *GST-T1* gene absence and better toxicity prognosis and the lower (rather than higher) frequency of the low-activity *GST-P1* variant in intoxicated patients were unexpected, indicating that GST-T1 and GST-P1 may not be directly

involved in NAPQI detoxification through enzymatic conjugation with glutathione. In support of this, a recent in vitro study indicated that the *GST-P1* rs1695 amino acid variant had no effect on the rate of glutathione conjugation of NAPQI (Dragovic et al. 2014). Consequently the apparent protective effect of *GST-T1* deletion and reduced GST-P1 activity is likely related to mechanisms other than NAPQI conjugation by these enzymes. Interestingly, a study of mice with genetic deletion of the *GST-P1* gene unexpectedly also showed reduced sensitivity to APAP-induced hepatotoxicity that was associated with faster recovery of hepatic glutathione concentrations (Henderson et al. 2000). However a specific mechanism has not yet been elucidated.

The association of known and novel (by direct sequencing) variants in coding regions of the keratin (*KRT*) 8 and 18 genes with ALF risk and outcome in 344 patients enrolled in the ALFSG US multicenter observational study has been reported (Strnad et al. 2010). Of these, 167 patients had APAP-induced ALF. *KRT8* and *KRT18* encode proteins that provide antiapoptotic, cytoprotective effects during liver injury. The main finding of the study was a significantly higher allele frequency of the R341H variant in white patients with ALF (18/252, 7.1%) and the G434S variant in African American patients with ALF (10/41, 24%) compared with frequencies in ethnicity-/race-matched control subjects (23/727, 3.2%, and 25/245, 10%, respectively) for all causes of ALF. However, race-/ethnicity-matched comparisons of these genotypes were not provided for the APAP-induced ALF subgroup. The *KRT18* variants were much rarer and independent genotype associations could not be determined. However there was a trend ($P = 0.05$) for decreased spontaneous survival (without transplant) in multivariate analysis for APAP-induced ALF patients with nonconservative coding *KRT8* or *KRT18* variants. The finding for *KRT8* R341H was recently confirmed by showing that transgenic mice expressing the human *KRT8* 341H allele had enhanced sensitivity to APAP-induced hepatotoxicity compared with those expressing the human *KRT8* 341R wild-type allele (Guldiken et al. 2015).

Most recently, several studies have evaluated the association of selected polymorphisms in genes encoding the UGTs, SULTs, and CYPs that metabolize APAP with APAP-induced ALF in the white American ALFSG patients. In the first study, an SNP located in the 3′-untranslated region of the UGT1A gene (rs8330, c.2042C>G) was identified that was associated with an increased rate of APAP glucuronidation in human liver (Court et al. 2013). Through a series of in vitro studies, this SNP was shown to increase APAP glucuronidation by multiple UGT1A enzymes (including UGT1A1, UGT1A6, and UGT1A9 which share the same 3′-UTR) through enhanced fidelity of gene splicing and minimization of formation of UGT1A repressor protein variants (Court et al. 2013). Importantly, rs8330 was found at reduced allele frequency (16%) in patients with unintentional APAP-induced ALF when compared with ethnicity-/race-matched control populations (21–25%) (Fig. 4). However, the rs8330 allele frequency in ALF patients who had intentionally overdosed with APAP (26%) was similar to the control populations. This lack of association was postulated to reflect the higher acute dose of APAP consumed over a much shorter period in the intentional overdose patients that may have overwhelmed the capacity of the liver to glucuronidate APAP despite the presence of a high-enzyme activity allele.

A second study broadened the search to other polymorphisms in genes encoding the APAP-metabolizing enzymes UGT1A1, UGT1A6, UGT1A9, UGT2B15, SULT1A1, CYP2E1, and CYP3A5 (Court et al. 2014; Fig. 4). Of these only CYP3A5 rs776746 (c.216-237G > A) was associated with APAP-induced ALF. Specifically it was shown that intentional overdose APAP-induced ALF patients had a higher allele frequency (9%) compared with a race-/ethnicity-matched control population (4%) or patients with unintentional APAP-induced ALF (3%). In the same study (Court et al. 2014), polymorphisms were evaluated in several genes that have been associated with increased risk of APAP-induced hepatotoxicity in rodent

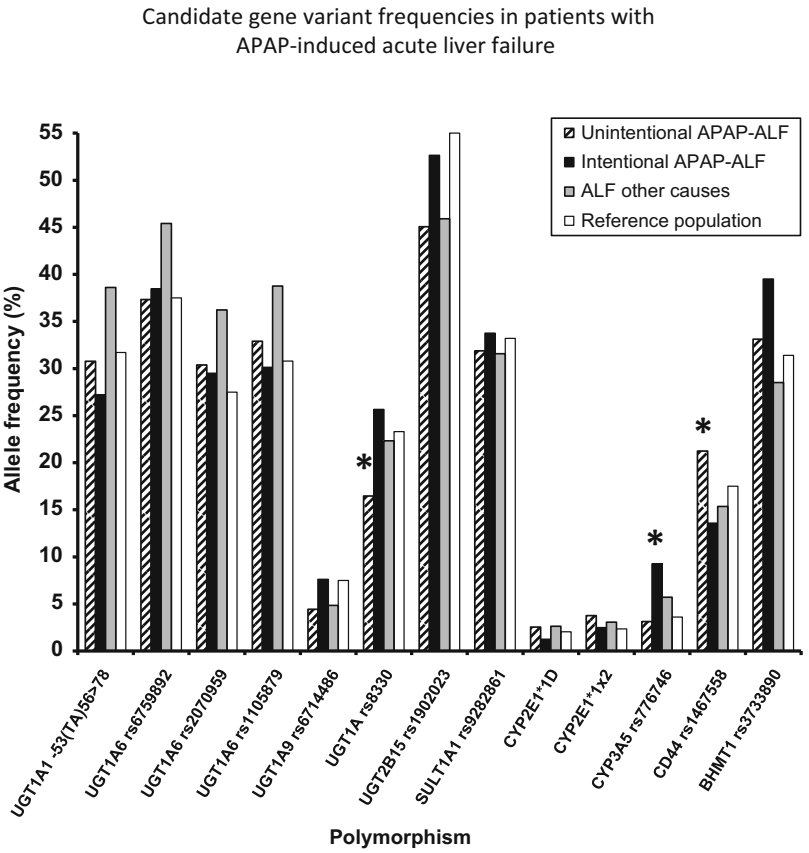

Fig. 4 Allele frequencies for candidate genes that may modify the risk for developing paracetamol (APAP)-induced acute liver failure (*ALF*). Shown are the allele frequencies for 261 white American patients who had developed ALF either unintentionally from chronic APAP use ($N = 79$), intentionally from a single time-point APAP overdose ($N = 78$), or from causes other than APAP ($N = 103$). Chi-squared analysis showed allele frequencies were different ($^*P < 0.05$) in patients with ALF who intentionally APAP overdosed (CYP3A5 rs776746) or patients with ALF who unintentionally overdosed (*UGT1A* rs8330 and *CD44* rs1467558) when compared with patients with ALF from other causes and also a race-/ethnicity-matched population

models, including *CD44* (rs1467558, c.689G>A, p.Ile479Thr) and *BHMT1* (rs3733890, c.716G>A, p.Arg239Gln). Although no association was found for the *BHMT1* variant, the *CD44* rs1467558 SNP showed a higher allele frequency in unintentional APAP-induced ALF patients (21%) compared with a matched control population (13%) and with intentional APAP-induced ALF patients (14%). This latter finding is consistent with the results of a previous study that found a significant association between the same *CD44* SNP (rs1467558) and mild persistent elevations (for more than 2 weeks) in serum alanine aminotransferase (ALT, a marker of hepatocellular injury) activity in volunteers who received the maximum recommended dose of APAP (4 g per day) for 7–14 days (Watkins et al. 2006; Harrill et al. 2009; Fig. 5). However it was unclear whether patients would have developed fulminant liver toxicity if dosing had been continued for longer than 2 weeks. A more recent study (Heard et al. 2014) addressed this question by studying the effects of maximum recommended daily dosing of APAP to healthy volunteers for up to 40 days. That study also showed mild persistent serum ALT elevations in 48 of 205 subjects after 16 days of treatment, but showed ALT values returned to baseline values in 47 of 48 subjects after 40 days. Although one patient had ALT values that continued to rise after the end of dosing at 40 days (nearly three times of elevation over the baseline of study day 63), that subject was lost to follow-up. As yet, it is unclear whether these subjects had also been genotyped for *CD44* rs1467558 or any other candidate gene variant.

Finally, although not a candidate gene association study, the effects of glutathione synthetase (GSS) deficiency which reduces tissue glutathione concentrations in tissues on toxicity biomarkers have been evaluated in several patients with this disorder. GSS deficiency is a rare autosomal recessive genetic disorder caused by a variety of mutations and characterized by metabolic acidosis, hemolytic anemia, and 5-oxoprolinemia. Although APAP-induced hepatotoxicity has not been directly evaluated in affected individuals, a surrogate ex vivo model has been developed and used to examine the role of genetically induced glutathione depletion on APAP toxicity (Spielberg and Gordon 1981). Specifically, the model used lymphocytes obtained from patients with GSS deficiency that were treated with APAP metabolites generated from mouse liver microsome incubations. Several studies showed lower glutathione content and enhanced cytotoxicity and reduced response to *N*-acetylcysteine treatment (which requires GSS for conversion to glutathione) in lymphocytes from subjects who were either homozygous (Spielberg and Gordon 1981) or heterozygous (Spielberg 1985) for GSS deficiency. However, these mutations are relatively rare, and it is unclear whether polymorphisms located in the GSS gene are associated with altered GSS activity or APAP toxicity.

Genome-Wide Association Studies

Genome-wide case-control association studies have successfully identified genes and associated genetic variants associated with adverse drug reactions in human patients (Nelson et al. 2009). A major limitation of this approach is the potential for a high rate

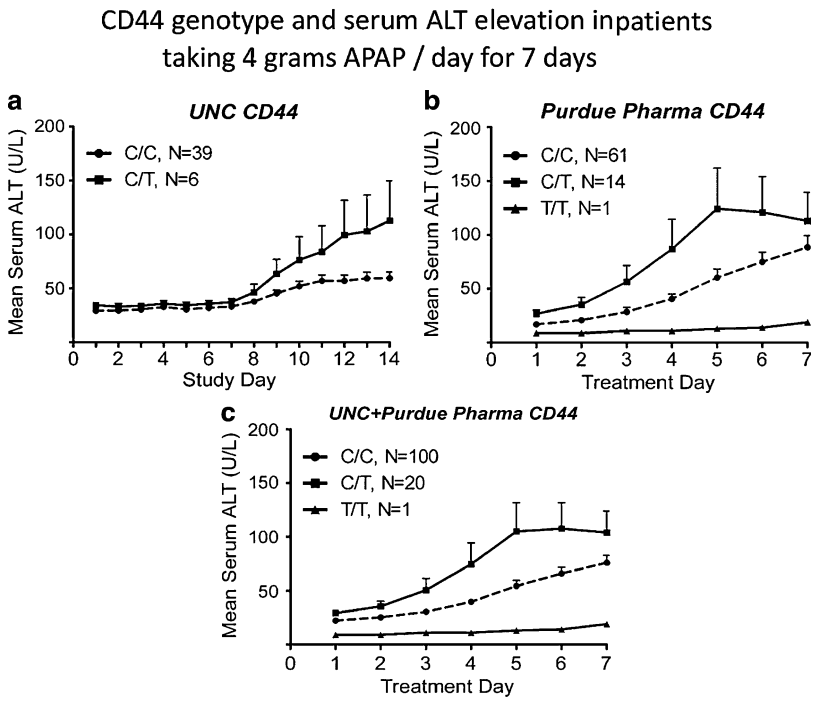

Fig. 5 Associations between *CD44* rs1467558 C/T genotype and changes in serum alanine aminotransferase (*ALT*) activity in healthy adult volunteers who received the maximum recommended dose of paracetamol (4 g/day) for (**a**) 7 days (University of North Carolina [UNC] study) and (**b**) 14 days (Purdue Pharma study) or for (**c**) the combined set of data. Weak but statistically significant associations were observed between genotype and ALT elevations in the UNC study ($P = 0.02$) and the Purdue Pharma study ($P = 0.01$), while a much stronger association ($P = 0.002$) was found for the combined data set (Originally published as Fig. 5 in Harrill et al. (2009) and reproduced with permission from Cold Spring Harbor Laboratory Press)

of false-positive associations given the large number of variants that can be evaluated (over 500,000), which requires evaluation of sufficient numbers of well-matched case and control subjects. However, the results of a recent analysis indicate that (strong) effects from a single gene can be identified with as few as 15 cases and 200 population controls (Nelson et al. 2009). Unfortunately, there have been no reports of genome-wide association studies evaluating risk or outcome for APAP-induced ALF.

Potential Applications to Prognosis, Other Diseases, or Conditions

As suggested by the candidate gene studies described above (Table 2), the identified genetic variants could be used to prospectively predict an individual patient's risk for developing ALF following acute or chronic overdose of APAP, the severity of the

symptoms (such as hepatic encephalopathy), and likelihood of possible outcomes (transplant, spontaneous recovery, or death). Specific uses of these biomarkers might include identifying patients at high risk of developing ALF from therapeutic use of APAP who should be advised to avoid or minimize excessive APAP use, determining the need for *N*-acetylcysteine antidote treatment following APAP overdose, and predicting whether aggressive symptomatic treatments such as liver transplant will be needed. However, given the influence of nongenetic factors (Table 1), these variants would need to be incorporated into a larger predictive algorithm that also includes such covariates as patient age, nutritional status, the presence of liver disease, alcohol use, and coadministered drugs. As suggested by the studies conducted by Court et al. (2013, 2014), this algorithm would also need to adjust for the type of APAP ingestion (unintentional multidose versus intentional single-point overdose). Serum APAP-protein adduct concentrations could help to differentiate these overdose types (Khandelwal et al. 2011). Other biomarkers measured in patient serum samples, such as concentrations of APAP, hepatocyte-specific enzymes (including alanine aminotransferase), and microRNAs, mitochondrial DNA, and various others currently under study, may also enhance predictive accuracy (McGill and Jaeschke 2014).

Gene variant biomarkers could also be incorporated into the current methods used to predict the need for *N*-acetylcysteine treatment, such as the Rumack-Matthew nomogram. This clinical tool uses admission serum APAP concentrations and time from overdose to admission to identify patients that require *N*-acetylcysteine treatment to prevent ALF (Khandelwal et al. 2011). Although serum APAP-protein adduct concentrations have also been proposed to enhance the accuracy of this approach, it is based on APAP exposure and does not identify patients that are more sensitive to a given APAP dose.

Finally, it is clear that relatively few candidate gene variants have been evaluated to date, and of those the associations with risk and outcome are relatively weak. It is possible that common and rare variants may exist in genes that are not obvious candidates in the APAP metabolism and hepatotoxicity pathway shown in Fig. 3. Consequently, unbiased approaches, such as whole-genome association studies, are needed to identify variants that could have a greater impact on toxicity risk and outcome than those evaluated to date.

Summary Points

- Paracetamol (APAP, also called acetaminophen) is one of the most widely used analgesic and antipyretic drugs in the world.
- APAP is also one of the most common causes of acute liver failure (ALF) in the USA and many other Western countries.
- In the USA, about half of the cases resulted from a single time-point APAP overdose with intention of self-harm, while the remaining cases were taking APAP for therapeutic purposes at high doses for days to weeks.

- Geographic differences in APAP-induced ALF incidence are not well understood but may be a consequence of availability, use in combination with opioids, prevalence of alcohol use, and population genetic differences.
- Nongenetic factors that increase risk include fasting, liver disease, and chronic alcohol use; concurrent use of some drugs, including the fibrates, nonsteroidal anti-inflammatories, statins, angiotensin-converting enzyme inhibitors, and angiotensin receptor II antagonists decreases risk.
- The main biomarkers currently used to predict risk for APAP-induced ALF after an overdose include serum APAP and APAP-protein adduct concentrations; serum concentrations of hepatocyte-specific alanine aminotransferase are also used to monitor the severity and course of hepatotoxicity.
- Variants in genes involved in the pathogenesis of APAP-induced ALF could be used as predictive biomarkers to determine an individual's risk for developing APAP-induced ALF, need for specific therapies (*N*-acetylcysteine and liver transplant), and likelihood of recovery.
- Candidate genes that have been associated with altered risk and/or outcome of APAP-induced ALF included *UGT1A*, *CD44*, *CYP3A5*, *GST-P1*, *GST-T1*, *KRT8*, and *TLA*.
- For some genes the associations were dependent on whether the APAP overdose was acute intentional (*CYP3A5*) or chronic unintentional (*UGT1A* and *CD44*).
- Additional candidate gene and whole-genome studies are needed to identify the major genes and gene variants associated with APAP-induced ALF.

References

Adukauskiene D, Dockiene I, Naginiene R, Kevelaitis E, Pundzius J, Kupcinskas L. Acute liver failure in Lithuania. Medicina (Kaunas). 2008;44:536–40.

Ameer B, Greenblatt DJ. Acetaminophen. Ann Intern Med. 1977;87:202–9.

Bailey DN, Briggs JR. The binding of selected therapeutic drugs to human serum alpha-1 acid glycoprotein and to human serum albumin in vitro. Ther Drug Monit. 2004;26:40–3.

Bernal W, Donaldson P, Underhill J, Wendon J, Williams R. Tumor necrosis factor genomic polymorphism and outcome of acetaminophen (paracetamol)-induced acute liver failure. J Hepatol. 1998;29:53–9.

Boess F, Bopst M, Althaus R, Polsky S, Cohen SD, Eugster HP, Boelsterli UA. Acetaminophen hepatotoxicity in tumor necrosis factor/lymphotoxin-alpha gene knockout mice. Hepatology. 1998;27:1021–9.

Bretherick AD, Craig DG, Masterton G, Bates C, Davidson J, Martin K, Iredale JP, Simpson KJ. Acute liver failure in Scotland between 1992 and 2009; incidence, aetiology and outcome. QJM. 2011;104:945–56.

Buchard A, Eefsen M, Semb S, Andersen SE, Morling N, Bendtsen F, Larsen FS, Dalhoff K. The role of the glutathione S-transferase genes GSTT1, GSTM1, and GSTP1 in acetaminophen-poisoned patients. Clin Toxicol (Phila). 2012;50:27–33.

Butterworth RF. Pathogenesis of hepatic encephalopathy and brain edema in acute liver failure. J Clin Exp Hepatol. 2015;5:S96–103.

Chan TY. The epidemiology of acetaminophen (paracetamol) poisoning in Hong Kong. Vet Hum Toxicol. 1996;38:443–4.

Chen C, Hennig GE, Whiteley HE, Corton JC, Manautou JE. Peroxisome proliferator-activated receptor alpha-null mice lack resistance to acetaminophen hepatotoxicity following clofibrate exposure. Toxicol Sci. 2000;57:338–44.

Cheng J, Ma X, Krausz KW, Idle JR, Gonzalez FJ. Rifampicin-activated human pregnane X receptor and CYP3A4 induction enhance acetaminophen-induced toxicity. Drug Metab Dispos. 2009;37:1611–21.

Coles B, Wilson I, Wardman P, Hinson JA, Nelson SD, Ketterer B. The spontaneous and enzymatic reaction of *N*-acetyl-p-benzoquinonimine with glutathione: a stopped-flow kinetic study. Arch Biochem Biophys. 1988;264:253–60.

Court MH, Freytsis M, Wang X, Peter I, Guillemette C, Hazarika S, Duan SX, Greenblatt DJ, Lee WM, Acute Liver Failure Study G. The UDP-glucuronosyltransferase (UGT) 1A polymorphism c.2042C>G (rs8330) is associated with increased human liver acetaminophen glucuronidation, increased UGT1A exon 5a/5b splice variant mRNA ratio, and decreased risk of unintentional acetaminophen-induced acute liver failure. J Pharmacol Exp Ther. 2013;345:297–307.

Court MH, Peter I, Hazarika S, Vasiadi M, Greenblatt DJ, Lee WM, Acute Liver Failure Study G. Candidate gene polymorphisms in patients with acetaminophen-induced acute liver failure. Drug Metab Dispos. 2014;42:28–32.

Dordoni B, Willson RA, Thompson RP, Williams R. Reduction of absorption of paracetamol by activated charcoal and cholestyramine: a possible therapeutic measure. Br Med J. 1973; 3:86–7.

Dragovic S, Venkataraman H, Begheijn S, Vermeulen NP, Commandeur JN. Effect of human glutathione S-transferase hGSTP1-1 polymorphism on the detoxification of reactive metabolites of clozapine, diclofenac and acetaminophen. Toxicol Lett. 2014;224:272–81.

Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T, Harada T, Yamamoto M. High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. Toxicol Sci. 2001;59:169–77.

Gelotte CK, Auiler JF, Lynch JM, Temple AR, Slattery JT. Disposition of acetaminophen at 4, 6, and 8 g/day for 3 days in healthy young adults. Clin Pharmacol Ther. 2007;81:840–8.

Gow PJ, Jones RM, Dobson JL, Angus PW. Etiology and outcome of fulminant hepatic failure managed at an Australian liver transplant unit. J Gastroenterol Hepatol. 2004;19:154–9.

Gregory B, Larson AM, Reisch J, Lee WM, Acute Liver Failure Study G. Acetaminophen dose does not predict outcome in acetaminophen-induced acute liver failure. J Investig Med. 2010;58:707–10.

Guldiken N, Zhou Q, Kucukoglu O, Rehm M, Levada K, Gross A, Kwan R, James LP, Trautwein C, Omary MB, Strnad P. Human keratin 8 variants promote mouse acetaminophen hepatotoxicity coupled with c-jun amino-terminal kinase activation and protein adduct formation. Hepatology. 2015;62:876–86.

Gunawan BK, Liu ZX, Han D, Hanawa N, Gaarde WA, Kaplowitz N. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. Gastroenterology. 2006;131:165–78.

Harrill AH, Watkins PB, Su S, Ross PK, Harbourt DE, Stylianou IM, Boorman GA, Russo MW, Sackler RS, Harris SC, Smith PC, Tennant R, Bogue M, Paigen K, Harris C, Contractor T, Wiltshire T, Rusyn I, Threadgill DW. Mouse population-guided resequencing reveals that variants in CD44 contribute to acetaminophen-induced liver injury in humans. Genome Res. 2009;19:1507–15.

Hazai E, Vereczkey L, Monostory K. Reduction of toxic metabolite formation of acetaminophen. Biochem Biophys Res Commun. 2002;291:1089–94.

Heard K, Green JL, Anderson V, Bucher-Bartelson B, Dart RC. A randomized, placebo-controlled trial to determine the course of aminotransferase elevation during prolonged acetaminophen administration. BMC Pharmacol Toxicol. 2014;15:39.

Henderson CJ, Wolf CR, Kitteringham N, Powell H, Otto D, Park BK. Increased resistance to acetaminophen hepatotoxicity in mice lacking glutathione S-transferase Pi. Proc Natl Acad Sci U S A. 2000;97:12741–5.

Hjelle JJ. Hepatic UDP-glucuronic acid regulation during acetaminophen biotransformation in rats. J Pharmacol Exp Ther. 1986;237:750–6.
Jaeschke H, Williams CD, Ramachandran A, Bajt ML. Acetaminophen hepatotoxicity and repair: the role of sterile inflammation and innate immunity. Liver Int. 2012;32:8–20.
Jaeschke H, Xie Y, McGill MR. Acetaminophen-induced liver injury: from animal models to humans. J Clin Transl Hepatol. 2014;2:153–61.
Javitt NB, Lee YC, Shimizu C, Fuda H, Strott CA. Cholesterol and hydroxycholesterol sulfotransferases: identification, distinction from dehydroepiandrosterone sulfotransferase, and differential tissue expression. Endocrinology. 2001;142:2978–84.
Kaufman DW, Kelly JP, Rosenberg L, Anderson TE, Mitchell AA. Recent patterns of medication use in the ambulatory adult population of the United States: the Slone survey. JAMA. 2002;287:337–44.
Khandelwal N, James LP, Sanders C, Larson AM, Lee WM, Acute Liver Failure Study G. Unrecognized acetaminophen toxicity as a cause of indeterminate acute liver failure. Hepatology. 2011;53:567–76.
Laine JE, Auriola S, Pasanen M, Juvonen RO. Acetaminophen bioactivation by human cytochrome P450 enzymes and animal microsomes. Xenobiotica. 2009;39:11–21.
Lancaster EM, Hiatt JR, Zarrinpar A. Acetaminophen hepatotoxicity: an updated review. Arch Toxicol. 2015;89:193–9.
Larson AM, Polson J, Fontana RJ, Davern TJ, Lalani E, Hynan LS, Reisch JS, Schiodt FV, Ostapowicz G, Shakil AO, Lee WM, Acute Liver Failure Study G. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. Hepatology. 2005;42:1364–72.
Lee WM. Acute liver failure. Semin Respir Crit Care Med. 2012;33:36–45.
Lee S, Dawson PA, Hewavitharana AK, Shaw PN, Markovich D. Disruption of NaS1 sulfate transport function in mice leads to enhanced acetaminophen-induced hepatotoxicity. Hepatology. 2006;43:1241–7.
Lee FY, de Aguiar Vallim TQ, Chong HK, Zhang Y, Liu Y, Jones SA, Osborne TF, Edwards PA. Activation of the farnesoid X receptor provides protection against acetaminophen-induced hepatic toxicity. Mol Endocrinol. 2010;24:1626–36.
Leonis MA, Alonso EM, Im K, Belle SH, Squires RH, Pediatric Acute Liver Failure Study G. Chronic acetaminophen exposure in pediatric acute liver failure. Pediatrics. 2013;131: e740–6.
Liu J, Wu KC, Lu YF, Ekuase E, Klaassen CD. Nrf2 protection against liver injury produced by various hepatotoxicants. Oxid Med Cell Longev. 2013;2013:305861.
Manautou JE, de Waart DR, Kunne C, Zelcer N, Goedken M, Borst P, Elferink RO. Altered disposition of acetaminophen in mice with a disruption of the Mrp3 gene. Hepatology. 2005;42:1091–8.
Manthripragada AD, Zhou EH, Budnitz DS, Lovegrove MC, Willy ME. Characterization of acetaminophen overdose-related emergency department visits and hospitalizations in the United States. Pharmacoepidemiol Drug Saf. 2011;20:819–26.
Manyike PT, Kharasch ED, Kalhorn TF, Slattery JT. Contribution of CYP2E1 and CYP3A to acetaminophen reactive metabolite formation. Clin Pharmacol Ther. 2000;67:275–82.
Mazaleuskaya LL, Sangkuhl K, Thorn CF, FitzGerald GA, Altman RB, Klein TE. PharmGKB summary: pathways of acetaminophen metabolism at the therapeutic versus toxic doses. Pharmacogenet Genomics. 2015;25:416–26.
McGill MR, Jaeschke H. Metabolism and disposition of acetaminophen: recent advances in relation to hepatotoxicity and diagnosis. Pharm Res. 2013;30:2174–87.
McGill MR, Jaeschke H. Mechanistic biomarkers in acetaminophen-induced hepatotoxicity and acute liver failure: from preclinical models to patients. Expert Opin Drug Metab Toxicol. 2014;10:1005–17.

McGill MR, Sharpe MR, Williams CD, Taha M, Curry SC, Jaeschke H. The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. J Clin Invest. 2012;122:1574–83.

Mendizabal M, Marciano S, Videla MG, Anders M, Zerega A, Balderramo DC, Chan D, Barrabino M, Gil O, Mastai R, Yantorno S, Gadano A, Silva MO. Changing etiologies and outcomes of acute liver failure: perspectives from 6 transplant centers in Argentina. Liver Transpl. 2014;20:483–9.

Michaut A, Moreau C, Robin MA, Fromenty B. Acetaminophen-induced liver injury in obesity and nonalcoholic fatty liver disease. Liver Int. 2014;34:e171–9.

Mitchell JR, Thorgeirsson SS, Potter WZ, Jollow DJ, Keiser H. Acetaminophen-induced hepatic injury: protective role of glutathione in man and rationale for therapy. Clin Pharmacol Ther. 1974;16:676–84.

Mutlib AE, Goosen TC, Bauman JN, Williams JA, Kulkarni S, Kostrubsky S. Kinetics of acetaminophen glucuronidation by UDP-glucuronosyltransferases 1A1, 1A6, 1A9 and 2B15. Potential implications in acetaminophen-induced hepatotoxicity. Chem Res Toxicol. 2006;19:701–9.

Myers RP, Shaheen AA, Li B, Dean S, Quan H. Impact of liver disease, alcohol abuse, and unintentional ingestions on the outcomes of acetaminophen overdose. Clin Gastroenterol Hepatol. 2008;6:918–25. quiz 837.

Nelson MR, Bacanu SA, Mosteller M, Li L, Bowman CE, Roses AD, Lai EH, Ehm MG. Genome-wide approaches to identify pharmacogenetic contributions to adverse drug reactions. Pharmacogenomics J. 2009;9:23–33.

Okawa H, Motohashi H, Kobayashi A, Aburatani H, Kensler TW, Yamamoto M. Hepatocyte-specific deletion of the keap1 gene activates Nrf2 and confers potent resistance against acute drug toxicity. Biochem Biophys Res Commun. 2006;339:79–88.

Ostapowicz G, Lee WM. Acute hepatic failure: a Western perspective. J Gastroenterol Hepatol. 2000;15:480–8.

Patten CJ, Thomas PE, Guy RL, Lee M, Gonzalez FJ, Guengerich FP, Yang CS. Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. Chem Res Toxicol. 1993;6:511–8.

Polson J, Lee WM. Etiologies of acute liver failure: location, location, location! Liver Transpl. 2007;13:1362–3.

Possamai LA, McPhail MJ, Quaglia A, Zingarelli V, Abeles RD, Tidswell R, Puthucheary Z, Rawal J, Karvellas CJ, Leslie EM, Hughes RD, Ma Y, Jassem W, Shawcross DL, Bernal W, Dharwan A, Heaton ND, Thursz M, Wendon JA, Mitry RR, Antoniades CG. Character and temporal evolution of apoptosis in acetaminophen-induced acute liver failure. Crit Care Med. 2013;41:2543–50.

Prescott LF. Kinetics and metabolism of paracetamol and phenacetin. Br J Clin Pharmacol. 1980;10 Suppl 2:291S–8.

Randolph AG, Lange C, Silverman EK, Lazarus R, Weiss ST. Extended haplotype in the tumor necrosis factor gene cluster is associated with asthma and asthma-related phenotypes. Am J Respir Crit Care Med. 2005;172:687–92.

Riches Z, Stanley EL, Bloomer JC, Coughtrie MW. Quantitative evaluation of the expression and activity of five major sulfotransferases (SULTs) in human tissues: the SULT "pie". Drug Metab Dispos. 2009;37:2255–61.

Rodriguez Lopez M, Perez Saborido B, Pacheco Sanchez D, Asensio Diaz E, Labarga Rodriguez F, Martinez Diaz R, Gonzalo Martin M, Velasco Lopez R, Pinto Fuentes P, Barrera Rebollo A. Transplantation for acute liver failure: report of results in the region of Castilla y Leon (Spain) after 10 years of activity. Transplant Proc. 2012;44:2625–6.

Saini SP, Zhang B, Niu Y, Jiang M, Gao J, Zhai Y, Hoon Lee J, Uppal H, Tian H, Tortorici MA, Poloyac SM, Qin W, Venkataramanan R, Xie W. Activation of liver X receptor increases acetaminophen clearance and prevents its toxicity in mice. Hepatology. 2011;54:2208–17.

Sakakibara Y, Yanagisawa K, Katafuchi J, Ringer DP, Takami Y, Nakayama T, Suiko M, Liu MC. Molecular cloning, expression, and characterization of novel human SULT1C sulfotransferases that catalyze the sulfonation of *N*-hydroxy-2-acetylaminofluorene. J Biol Chem. 1998;273:33929–35.

Siegers CP, Loeser W, Gieselmann J, Oltmanns D. Biliary and renal excretion of paracetamol in man. Pharmacology. 1984;29:301–3.

Singhal S, Chakravarty A, Das BC, Kar P. Tumour necrosis factor-alpha and soluble Fas ligand as biomarkers in non-acetaminophen-induced acute liver failure. Biomarkers. 2009;14:347–53.

Spielberg SP. Acetaminophen toxicity in lymphocytes heterozygous for glutathione synthetase deficiency. Can J Physiol Pharmacol. 1985;63:468–71.

Spielberg SP, Gordon GB. Glutathione synthetase-deficient lymphocytes and acetaminophen toxicity. Clin Pharmacol Ther. 1981;29:51–5.

Strnad P, Zhou Q, Hanada S, Lazzeroni LC, Zhong BH, So P, Davern TJ, Lee WM, Acute Liver Failure Study G, Omary MB. Keratin variants predispose to acute liver failure and adverse outcome: race and ethnic associations. Gastroenterology. 2010;139:828–35. 835 e821-823.

Stuber F, Petersen M, Bokelmann F, Schade U. A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-alpha concentrations and outcome of patients with severe sepsis. Crit Care Med. 1996;24:381–4.

Suzuki A, Yuen N, Walsh J, Papay J, Hunt CM, Diehl AM. Co-medications that modulate liver injury and repair influence clinical outcome of acetaminophen-associated liver injury. Clin Gastroenterol Hepatol. 2009;7:882–8.

Thummel KE, Lee CA, Kunze KL, Nelson SD, Slattery JT. Oxidation of acetaminophen to *N*-acetyl-p-aminobenzoquinone imine by human CYP3A4. Biochem Pharmacol. 1993;45:1563–9.

Vredenburg G, Elias NS, Venkataraman H, Hendriks DF, Vermeulen NP, Commandeur JN, Vos JC. Human NAD(P)H:quinone oxidoreductase 1 (NQO1)-mediated inactivation of reactive quinoneimine metabolites of diclofenac and mefenamic acid. Chem Res Toxicol. 2014;27:576–86.

Watkins PB, Kaplowitz N, Slattery JT, Colonese CR, Colucci SV, Stewart PW, Harris SC. Aminotransferase elevations in healthy adults receiving 4 grams of acetaminophen daily: a randomized controlled trial. JAMA. 2006;296:87–93.

Wei G, Bergquist A, Broome U, Lindgren S, Wallerstedt S, Almer S, Sangfelt P, Danielsson A, Sandberg-Gertzen H, Loof L, Prytz H, Bjornsson E. Acute liver failure in Sweden: etiology and outcome. J Intern Med. 2007;262:393–401.

Whitcomb DC, Block GD. Association of acetaminophen hepatotoxicity with fasting and ethanol use. JAMA. 1994;272:1845–50.

Wu Y, Zhang X, Bardag-Gorce F, Robel RC, Aguilo J, Chen L, Zeng Y, Hwang K, French SW, Lu SC, Wan YJ. Retinoid X receptor alpha regulates glutathione homeostasis and xenobiotic detoxification processes in mouse liver. Mol Pharmacol. 2004;65:550–7.

Xie Y, McGill MR, Dorko K, Kumer SC, Schmitt TM, Forster J, Jaeschke H. Mechanisms of acetaminophen-induced cell death in primary human hepatocytes. Toxicol Appl Pharmacol. 2014;279:266–74.

Xie Y, Ramachandran A, Breckenridge DG, Liles JT, Lebofsky M, Farhood A, Jaeschke H. Inhibitor of apoptosis signal-regulating kinase 1 protects against acetaminophen-induced liver injury. Toxicol Appl Pharmacol. 2015;286:1–9.

Yamamoto A, Liu MY, Kurogi K, Sakakibara Y, Saeki Y, Suiko M, Liu MC. Sulphation of acetaminophen by the human cytosolic sulfotransferases: a systematic analysis. J Biochem. 2015;158:497–504.

Zamek-Gliszczynski MJ, Hoffmaster KA, Tian X, Zhao R, Polli JW, Humphreys JE, Webster LO, Bridges AS, Kalvass JC, Brouwer KL. Multiple mechanisms are involved in the biliary excretion of acetaminophen sulfate in the rat: role of Mrp2 and Bcrp1. Drug Metab Dispos. 2005;33:1158–65.

Zamek-Gliszczynski MJ, Nezasa K, Tian X, Bridges AS, Lee K, Belinsky MG, Kruh GD, Brouwer KL. Evaluation of the role of multidrug resistance-associated protein (Mrp) 3 and Mrp4 in

hepatic basolateral excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in Abcc3-/- and Abcc4-/- mice. J Pharmacol Exp Ther. 2006a;319:1485–91.

Zamek-Gliszczynski MJ, Nezasa K, Tian X, Kalvass JC, Patel NJ, Raub TJ, Brouwer KL. The important role of Bcrp (Abcg2) in the biliary excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in mice. Mol Pharmacol. 2006b;70:2127–33.

Zhang J, Huang W, Chua SS, Wei P, Moore DD. Modulation of acetaminophen-induced hepatotoxicity by the xenobiotic receptor CAR. Science. 2002;298:422–4.

Zhao P, Wang C, Liu W, Chen G, Liu X, Wang X, Wang B, Yu L, Sun Y, Liang X, Yang H, Zhang F. Causes and outcomes of acute liver failure in China. PLoS One. 2013;8, e80991.

PNPLA3 Polymorphism and Nonalcoholic Fatty Liver Disease

32

Olena Kolesnikova, Valeriya Nemtsova, and Rajkumar Rajendram

Contents

O. Kolesnikova (✉)
Department of Hepatology, Government Institution L.T. Malaya Therapy, National Institute of NAMS of Ukraine, Kharkiv, Ukraine
e-mail: kolesnikova1973@gmail.com; igor@mast.kharkov.ua

V. Nemtsova
Clinical Pharmacology Department, Kharkov National Medical University, Kharkiv, Ukraine
e-mail: valeriyan@ukr.net

R. Rajendram
Diabetes and Nutritional Sciences Research Division, Faculty of Life Science and Medicine, School of Biomedical and Health Sciences, King's College London, London, UK

Department of Internal Medicine, King Abdulaziz Medical City, National Guard Hospital Affairs, Riyadh, Saudi Arabia
e-mail: rajkumarrajendram@doctors.org.uk

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_28

Abstract

Nonalcoholic fatty liver disease (NAFLD) is an independent risk factor for metabolic disorders and cardiovascular events. Patients with metabolic disorders are also predisposed to the development of NAFLD and are at increased risk of cardiovascular events. Management of modifiable cardiovascular risk factors is therefore important in patients with NAFLD.

Recent advances have increased the understanding of the role of single nucleotide polymorphisms in the pathogenesis, risk stratification, and treatment of NAFLD. One of the most important genes in the development of NAFLD is *patatin-like phospholipase domain containing 3* (*PNPLA3*). Progression of hepatic steatosis has been associated with carriage of allele G of polymorphism rs738409 of *PNPLA3*. NAFLD patients with genotype GG rs738409 *PNPLA3* have more severe hepatic steatosis than heterozygotes and those without the G allele. Also patients with NAFLD who carried allele G rs738409 *PNPLA3* had a proatherogenic lipid profile and increases thickness of the carotid artery intima-media and level of cardiovascular risk.

Genetic factors and metabolic phenotypes influence liver fat content. These characteristics also influence cardiovascular risk. This is probably due to the effects of these genotypes and phenotypes on lipid receptors in the liver. For example, polymorphisms in *PNPLA3* influence the level of expression of *PNPLA3* and/or the function of the associated protein PNPLA3 (also known as adiponutrin-3). Study of *PNPLA3* polymorphisms has increased our understanding of the pathogenesis of liver disease. These polymorphisms are biomarkers which could be used to guide the use of medical therapies that prevent or treat liver disease.

Keywords

Nonalcoholic fatty liver disease • Gene polymorphism PNPLA3 liver fat content • Cardiovascular risk • Genetic determination

List of Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BFM	Body fat mass
BMI	Body mass index
CRP	C-reactive protein
CT	Computer tomography
DM-2	Type 2 diabetes mellitus
DNA	Deoxyribonucleic acid
DPI	Duplex-perfusion index
GGT	Gamma-glutamyltranspeptidase
HA	Hyaluronic acid

Hb_{1C}	Glycosylated hemoglobin
HDL	High-density lipoprotein
HRI	Hepatorenal index
IMT	Intima-media thickness
IR	Insulin resistance
LFC	Liver fat content
NAFLD	Nonalcoholic fatty liver disease
p.n.	Paired nucleotides
PCR	Polymerase chain reaction
PNPLA3	Adiponutrin gene
SAT	Subcutaneous fat
SNP	Single nucleotide polymorphisms
TC	Total cholesterol
TG	Triglycerides
TIMP-1	Tissue inhibitor of matrix metalloproteinase-1
TNF – α	Tumor necrosis factor-α
VAT	Visceral fat
VLDL	Very-low-density lipoproteins

Key Facts on the PNPLA3 Polymorphism and Nonalcoholic Fatty Liver Disease

Key facts on NAFLD:

- Nonalcoholic fatty liver disease (NAFLD) – an independent disease that includes a range of morphological changes in the liver parenchyma, steatosis (fatty liver), nonalcoholic steatohepatitis (NASH) that can lead to the development of the terminal stages of the disease: cirrhosis and liver cancer.
- Patients with NAFLD are usually asymptomatic. The diagnosis is usually made incidentally after laboratory and/or radiological investigation.
- The term NASH was first coined in 1980 when morphological studies found characteristic histological features of alcoholic liver disease in obese patients and patients with type 2 diabetes mellitus but no history of alcohol misuse.
- In the general population of Western countries, the overall prevalence of NAFLD is estimated to be 20–30%.
- Although NAFLD occurs in men and women of all ages, the incidence is highest in 40–60-year-old women with signs of metabolic syndrome.
- The prevalence of NAFLD increases with age, social and economic differences, and lifestyle.
- Polymorphism of genes involved in the regulation of lipid and carbohydrate metabolism is currently thought to play an important role in the pathogenesis of NAFLD.

Key facts on the *patatin-like phospholipase domain containing 3* (*PNPLA3*) gene:

- To date over 1060 genes, mainly associated with metabolism of fat in the liver, have been identified, 419 of which are associated with liver steatosis.
- The polymerase chain reaction (PCR) is used to study genes associated with predisposition to fatty liver disease.
- Adiponutrin is a non-secreted transmembrane protein predominantly expressed in brown and white adipose tissue that is derived from the *PNPLA3* gene. It is also expressed in the liver. A role in intracellular energy balance is suggested by the effect of calorific intake on the expression of its messenger RNA (mRNA) in adipose tissue.
- Identification of the effect of adiponutrin on lipoprotein metabolism initiated the study of the role of the associated gene *PNPLA3* in hepatic steatosis.
- *PNPLA3* gene polymorphism, which affects adiponutrin protein synthesis, results in reduction of triacylglycerol hydrolase activity and increased concentrations of triglycerides as a result of reduction of diglycerides.
- Over 70% of the genetic predisposition to NAFLD is caused by the GG genotype of the SNP rs738409 *PNPLA3*.

Definitions of Words and Terms

Apolipoprotein B 100	Apolipoprotein B (apo B) is an essential component of very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), intermediate density lipoprotein, and chylomicrons. Structural features of apo B100 perform the role of the LDL receptor ligand for hepatocytes and scavenger receptor of macrophages and endothelial cells.
Calcium-independent phospholipase A2	LP-PLA2 belongs to the family of phospholipase A2. It is produced in monocytes, mast cells, Kupffer cells, and T lymphocytes. In plasma, 80% LP-PLA2 is associated with low-density lipoproteins; the remaining 20% is bound to high-density lipoproteins and very-low-density lipoproteins. LP-PLA2 is an important indicator of cardiovascular risk. It is applicable to reclassification of the risk in patients groups with middle- and high-risk cardiovascular events.
Level of cardiovascular risk	Cardiovascular risk is the probability of developing cardiovascular events associated with

	atherosclerosis in a certain time period. To facilitate clinical management, cardiovascular risk can be stratified into four levels; very high, high, moderate, and low. Stratification of risk in this way guides the initiation of treatment to reduce risk factors on the basis of a risk/benefit ratio.
NAFLD liver fat score	Scale which allows quantification content of fat in the liver as a percentage.
Systematic COronary Risk Evaluation (SCORE) scale	A cardiovascular risk assessment tool commonly used in Europe. It stratifies patients with cardiovascular disease into low- and high-risk groups. It has several advantages; a clear design that is easy to use, multi-account etiology of CVD, the calculation of the risk of death from cardiovascular disease as well as coronary heart disease, objectification of the concept of cardiovascular risk, and the unification of the concept of risk for doctors from different countries.
Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1)	It is a protein of the extracellular matrix. There have been conflicting reports on the relationship of TIMP-1 with fibrogenesis which are likely due to the lack of tissue specificity of this biomarker. It is however considered to be a direct marker of liver fibrosis. Measurement of TIMP-1 is used in some complex panels to detect the presence of liver fibrosis.
Hyaluronic acid	This is a noninvasive marker of liver fibrosis. Increases in hyaluronic acid levels correlate better with the histological degree of liver damage than conventional serum tests of liver damage including ALT activity/AST activity, alkaline phosphatase activity, and bilirubin concentration.
Duplex-perfusion index (DPI)	The ratio of the hepatic arterial blood flow to the total blood flow in the liver; DPI is determined by ultrasound. It correlates with the severity of liver fibrosis.
Intima-media thickness	The thickness of the intima-media complex (IMT) and plaque in the carotid arteries is used as a marker for the presence of cardiovascular diseases. It is a predictor of atherosclerosis and risk of stroke.

Introduction

Nonalcoholic fatty liver disease NAFLD is defined by hepatic fat infiltration that involves over 5% hepatocytes in the absence of excessive alcohol intake or any other demonstrable cause of liver disease (Younossi et al. 2011; Zhan et al. 2016). It is rapidly becoming the most common liver disease worldwide, and while initially thought to be benign, it is actually a major cause of morbidity and mortality. It includes a broad histological spectrum of disease ranging from simple hepatic steatosis (i.e., fatty liver) and nonalcoholic steatohepatitis (NASH) with or without fibrosis to cirrhosis with all its complications (i.e., portal hypertension, chronic liver failure, hepatocellular carcinoma).

The metabolic syndrome is a cluster of cardiovascular risk factors that includes at least three of five of obesity, hypertension, raised fasting blood glucose, high serum triglycerides, and serum low high-density lipoprotein (HDL). Metabolic syndrome, type 2 diabetes mellitus (DM), atherosclerosis, and NAFLD are strongly associated. Insulin resistance is important in the pathogenesis of both NAFLD and the metabolic syndrome. Therefore, it is not surprising that NAFLD is an independent risk factor both general and visceral obesity (Tilg and Moschen 2010).

In Western countries, the prevalence of NAFLD is 20–30% (Ratziu 2012). The prevalence of NAFLD is 80–90% in obese adults, 30–50% in diabetics, and up to 90% in patients with hyperlipidemia. The overall prevalence of NAFLD in children is 3–10%, but can be up to 70% in obese children (Hooper 2011).

Although the natural history of NAFLD is not well defined, most people with NAFLD do not develop NASH. While it is estimated that currently only 2–3% of the general population has NASH, the incidence of NAFLD is increasing with changes in lifestyle. It is important to diagnose and treat NAFLD early to prevent progression to NASH, liver cirrhosis, and hepatic carcinoma (Wong 2013).

The pathogenesis of NAFLD has not yet been fully elucidated but ultimately results in oxidation of free fatty acids and accumulation of fat in the liver. Adipose tissue which stores the excess energy produced from the metabolism of food is also an endocrine organ. Expansion of adipose tissue increases production of adipokines (Williams 2011). These bioactive substances interact with processes in several organs including the liver and can initiate chronic low-grade inflammation. Although the precise mechanisms are still unclear, dysregulated production or secretion of these adipokines caused by excess adipose tissue and adipose tissue dysfunction can contribute to the development of obesity-related metabolic diseases such as NAFLD (Zhan et al. 2016).

Studies of genetic polymorphism revealed that changes in the regulation of the accumulation of fat in the liver promote the development of NAFLD genes which have been implicated to include those associated with the distribution of lipids, lipoprotein metabolism (e.g., apolipoprotein C3), and adiponectin levels (Kollerits et al. 2009, 2010).

It is thought that genotyping patients could guide the prevention and treatment of NAFLD. However, precisely which genes and their polymorphisms influence the development and progression of NAFLD must first be determined. Therefore, the

assessment of the accumulation of fat in the livers of the carriers of various genes and their polymorphisms is important.

Patatin-Like Phospholipase Domain Containing 3 (PNPLA3) and NAFLD

One of the most important genes implicated in the development of NAFLD is *patatin-like phospholipase domain containing 3* (*PNPLA3*). This gene is located on chromosome 22 and encodes 481 amino acids which form the protein patatin-like phospholipase domain containing 3 (also known as adiponutrin-3; Romeo et al. 2008). This is a transmembrane protein that is highly expressed in adipose tissue. Although the highest concentration of adiponutrin is found in adipocytes, in white fat it is also expressed in the liver (Rae-Whitcombe et al. 2010).

All four currently known subtypes of adiponutrin [glutamine synthetase 2 (GS2; PNPLA4), GS2-like, PNPLA1, and PNPLA3] have lipolytic activity (Valenti et al. 2010a, b; Tian et al. 2010). Adiponutrin-3 is a multifunctional enzyme with triacylglycerol lipase and acylglycerol O-acyltransferase activities (Pingitore 2013; Sookoian et al. 2009; Kotronen et al. 2009a, b; Kantartzis et al. 2009).

Initial evidence of the role of *PNPLA* in the pathogenesis of NAFLD was obtained from a genome-wide survey of sequence variation including 9229 single nucleotide polymorphisms (SNP) in Hispanics, African Americans, and Caucasians, in the Dallas heart study (Romeo et al. 2008; Speliotes et al. 2010; Sookoian and Pirola 2011). Two general genomic investigations have found that *PNPLA3* polymorphism is associated with pathological changes in the liver (Yuan et al. 2008; Romeo et al. 2008).

Genome-based analysis of SNP found a link between the gene *PNPLA3* and changes in plasma levels of liver enzymes in the nonalcoholic population (Romeo et al. 2010a, b; Huang et al. 2010). Investigation of the role of adiponutrin in lipoprotein metabolism leads to the association of *PNPLA3* with hepatic steatosis.

Although *PNPLA3* gene polymorphisms are associated with hepatic lipid accumulation, the precise intracellular role of PNPLA3 in vivo is unclear (Kollerits et al. 2009, 2010; Speliotes et al. 2010; Valenti et al. 2010a, b; Wilson et al. 2006). It is thought to be involved in the balance between use of energy and storage of lipids in adipocytes as well as the regulation of lipid metabolism in liver cells (Pirazzi et al. 2012; Ruhanen et al. 2014). This hypothesis is supported by the influence of calorific intake on the expression of its messenger RNA (mRNA) in adipose tissue and hepatocytes. A low-calorie diet reduced adiponutrin mRNA expression by 56%. This effect was reversed by provision of adequate nutrition (Romeo et al. 2010a, b; Santoro et al. 2010; Petit et al. 2010). Furthermore, levels of *PNPLA3* mRNA increase in the adipocytes and hepatocytes of obese patients. However, while clearly associated with steatosis, increased PNPLA3 activity does not correlate with insulin resistance (Romeo et al. 2008; Kantartzis et al. 2009).

So studies of *PNPLA3* polymorphism were therefore performed. The G allele of SNP rs738409 of *PNPLA3* results in a change from isoleucine to methionine

(p.I148M) in adiponutrin-3 and is associated with lower serum levels of total cholesterol and LDL cholesterol (Valenti et al. 2010a, b; Wilson et al. 2006). Carriers of the G allele also have smaller adipocytes (Krawczyk et al. 2010). The size of adipocytes reflects the amount of lipid that can be stored in subcutaneous adipose tissue. A smaller subcutaneous adipose tissue stores may lead to ectopic fat accumulation in organs such as the liver (Speliotes et al. 2010). The G allele of rs738409 *PNPLA3* is also thought to influence liver fat content, develop NAFLD, and affect the activities of liver enzymes in plasma (Kotronen et al. 2009a, b; Sookoian and Pirola 2011; Valenti et al. 2012; Wong 2013).

Several mechanisms have been proposed by which this polymorphism could cause NAFLD. For example, an in vivo study on rodents has shown that the protein encoded by the 148 M allele is not fully functional (Speliotes 2009). The loss of triacylglycerol hydrolase activity of PNPLA3 would reduce triglyceride production and VLDL synthesis. This would then reduce serum levels of cholesterol and increase lipid accumulation in the liver. The mechanism by which reduction of triglyceride hydrolysis in hepatocytes results in steatosis is described in more detail below.

Lipids absorbed in the intestine bind apolipoprotein B 100 in the liver. This is followed by formation of very-low-density lipoprotein (VLDL). Adiponutrin increases the concentration of triglycerides by reducing diglycerides and other lipids. Triglycerides, cholesterol, and apolipoproteins are assembled into VLDL in the liver. Adiponutrin-3 affects hepatic VLDL secretion in humans (Farrell 2010, He 2010). The loss of lipase activity in the 148 M variant may impair VLDL production by reducing lipidation of apolipoprotein B100 (ApoB100) (Kollerits et al. 2009, 2010; Palmer et al. 2012). Reduction of VLDL formation would reduce transport of lipid in the blood promoting accumulation of lipid in the liver and resulting in steatohepatitis (Pirazzi et al. 2012).

The association of the G allele of rs738409 *PNPLA3* with hepatic fat content has been confirmed in a recent meta-analysis (Sookoian and Pirola 2011). Furthermore, the frequency of the G allele is significantly higher in NAFLD patients than healthy controls. One study found that the risk in Italians and British people with the genotype GG was almost 3.3 times higher than those without the G allele (Valenti et al. 2010a, b). The expression of *PNPLA3* also correlates significantly with BMI in obese children (Santoro et al. 2010).

However, the data on the relationship between the G allele of rs738409 *PNPLA3* and the development and severity of NAFLD are still somewhat contradictory. Inclusion of the presence of the GG genotype in the NAFLD liver fat score developed to calculate hepatic fat content, and diagnose NAFLD did not increase the sensitivity or specificity of the score. As the predictive value of the GG genotype did not exceed 1% (Johansson et al. 2008), this suggests that other genetic factors are important in the development of this disease (Wang et al. 2011). The development of metabolic events in patients with NAFLD is closely related to cardiovascular risk. The effect of adiponutrin-3 polymorphisms on cardiovascular risk also remains unclear.

The exact role of the I148M polymorphism of *PNPLA3* in the development of NAFLD, obesity, and cardiovascular risk is unclear. We therefore decided to investigate the association between the G allele of rs738409 *PNPLA3*, NAFLD, obesity, and cardiovascular risk.

Methodology of a Study of the Role of the SNP rs738409 in *PNPLA3* in the Development of NAFLD and Obesity

All procedures performed in this study were conducted in accordance with the Helsinki Declaration of the World Medical Association. The study protocol was approved by the Scientific Bioethics Committee (Ukraine). All patients gave written informed consent.

The study included 378 people with NAFLD (246 males, 132 females; mean age ± standard deviation, 42.6 ± 3.4 years). The control group consisted of 50 subjects with BMI <25 kg/m^2 without NAFLD, matched for age and sex with the subjects studied.

Patients were selected according to the following inclusion criteria:

Liver steatosis diagnosed by finding increased liver tissue echogenicity on ultrasound or computed tomography (CT) more than 3 months prior to the study
Obesity (body mass index (BMI) over 30 kg/m^2)
Type 2 diabetes mellitus
Hypertension
Dyslipidemia

Exclusion criteria included misuse of alcohol (consumption of <50 g ethanol/week for men, <30 g ethanol/week for women in the last year); chronic viral hepatitis (associated with HBV, HCV, or HDV infection); autoimmune, hereditary, or drug-induced hepatitis; previous myocardial infarction; coronary artery surgery; or stroke.

Basic anthropometric data (BMI and body fat mass; BFM) were collected. The distribution, mean, and standard deviations were calculated. A 4-component model was used to calculate the BFM which reflects the accumulation of fat tissue:

$$\mathrm{BFM} = 64.5 - 848/\mathrm{BMI} + 0.079 \bullet \mathrm{age} - 16.4 \bullet \mathrm{sex} + 0.05 \bullet \mathrm{Gender} \bullet \mathrm{Age} + 39.0 \bullet \mathrm{age}$$

where value assigned to gender is 0 for women and 1 for men. Age is measured in years.

The presence and severity of hepatic steatosis were assessed using the hepatorenal index (ratio of the density of the liver to the density of the renal parenchyma) on ultrasound, which was determined using a computer program. The duplex-perfusion index (DPI; ratio of blood flow in the hepatic artery to the

total blood flow in the liver) was measured using Doppler ultrasound (Kakkos et al. 2000; Mohammadinia et al. 2010).

Patients were then divided into groups by degree of hepatic steatosis:

1. Obese patients with NAFLD and liver steatosis degree I ($n = 140$)
2. Obese patients with NAFLD and hepatic steatosis degree II ($n = 150$)
3. Obese patients with NAFLD and hepatic steatosis degree III ($n = 88$)

In the second stage of the study, a computer tomography (CT) scan (HiSpeed CT/e Dual, General Electric, USA) was performed to assess the area of visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) at the level of the navel. The VAT/SAT ratio in the abdomen was measured in the axial plane at the center of lines connecting the lower edge of the costal arch with the anterior superior iliac spines using the method proposed by Park et al. (2008).

Lipid profile, markers of glycemic control, markers of liver damage, and adipose tissue hormone concentrations were measured in serum. Concentrations of total cholesterol (TC), high-density lipoprotein (HDL), and triglycerides (TG) were measured (enzymatic method, Humalyser autoanalyzer). Glycosylated hemoglobin (HbA1c) was measured using an immunoassay to assess long-term glycemic control. The serum markers of liver damage that were measured were aminotransferase (AST, ALT) activity, gamma-glutamyl transpeptidase (GGT) activity, alkaline phosphatase (ALP) activity, bilirubin concentration, thymol turbidity, serum protein concentration (albumin and gamma-globulins), and prothrombin index. Concentrations of adipose hormones were measured by ELISA on an immunoassay photometer analyzer (HumanReader); serum adiponectin (Jrgenium Laboratories Anti Biotech, Finland), resistin (Bio Vendor, Czech), and TNF-α (Biosource international, USA). Quantitative measurement of C-reactive protein (CRP) was also performed (DRG, USA).

Liver fat content (LFC) was calculated using the formula proposed (Kotronen et al. 2009a):

$$\begin{aligned} \mathrm{LFC} = 10(&-0.805 + 0.282 \bullet \text{metabolic syndrome (yes} = 1/\text{no} = 0) \\ &+ 0.078 \bullet \text{diabetes mellitus 2 type (yes} = 2/\text{no} = 0) \\ &+ 0.525 \bullet \text{Log (insulin mcU/L]).+ } 0.521 \bullet \text{Log ([AST])} \\ &- 0.454 \bullet \text{Log (AST/ALT))} \end{aligned}$$

Patients were then stratified into groups at high, moderate, or low risk of cardiovascular complications using the SCORE (Systematic COronary Risk Evaluation) scale. Measurement of the intima-media thickness (IMT) of the common carotid artery (CCA) was performed using ultrasound (Phillips IU, USA) by standard methodology (Stein 2008). Noninvasive markers of liver fibrosis, concentrations of hyaluronic acid (HA), and tissue inhibitor of metalloproteinase-1 (TIMP-1) were measured. Measurement of serum HA was performed by solid-phase enzyme analysis (Corgenix, USA), and measurement of TIMP-1 (Bio Source International, USA) was performed on an immunoassay photometer analyzer (HumaReader, HUMAN).

In the third stage of the study, the frequencies and genotypes of the SNP rs738409 of *PNPLA3* were determined in relation to the severity of hepatic steatosis and cardiovascular risk.

Molecular genetic studies of DNA. Genomic DNA was extracted from peripheral blood lymphocytes by a standard protocol (DIAtom ™ DNA Prep, Prep 200, Isogen Laboratory). This is the most effective method of DNA isolation within a short duration of extraction. It can isolate DNA from different biological materials and rapidly purifies DNA from clinical samples (e.g., whole blood, plasma, serum, urine, mucous membrane scrapings). The output of pure DNA from 200 μl of whole blood is 5–10 μg.

The principle of the DIAtom ™ DNA Prep kit is based on the use of a lysis agent with guanidine thiocyanate that destroys cells, solubilizes the cellular debris, and denatures cellular nucleases. In the presence of the lysis reagent, DNA is actively absorbed on NucleoS ™ sorbent. Salts and proteins are then washed away with an alcohol solution. Various methods can then be used to analyze the DNA obtained from the sorbent by Extra-Gene ™ or clean water.

The DNA genotyping was performed by tetra-primer polymerase chain reaction (PCR). This amplifies DNA fragments of different lengths corresponding to alternate alleles. Each exterior primer in combination with the corresponding inner primer initiates amplification of allele-specific fragments: for the SNP rs738409 of *PNPLA3*, 242 p.n.(normal) and 151 p.n.(mutation) were used.

Oligonucleotide primers (Table 1) for PCR were designed using software Vector NTI (Invitrogen) and NCBI information resource.

Table 1 Primer sequences of studied DNA polymorphisms

Genes-candidates	Polymorphic marker	Primers
PNPLA3 (adiponutrin-3)	rs 738409	*Internal*:
		PNPLA3 738409 R
		5′-TTGGTATGTTCCTGCTTCATC-3′
		PNPLA3 738409 F snp
		5′-ATAAGGCCACTGTAGAAGGGC-3′
		External:
		PNPLA3 738409 F
		5′-ACATGCAGTAAGTTTTGCTGCC-3′
		PNPLA3 738409 R
		5′-TTAACCTACTCTGTGCAAAGGG-3′
		snp[a]
		5′-TTGGTATGTTCCTGCTTCATG-3′

Design of oligonucleotide primers for PCR was performed using software Vector NTI (Invitrogen) and National Center for Biotechnology Information (NCBI) information resource. The obtained primers were tested for lack of complementarily between the 3′-ends to prevent the formation of primer dimers

[a]Additional primer with intentionally formed mismatch near the analyzed polymorphic site to improve the selectivity of enzyme-dependent reactions

Table 2 Temperature-time polymerase chain reaction regimes

Gene	Initiated denaturation	Denaturation	Primers annealing	Elongation	Final elongation	Volume of the reaction
PNPLA3 rs738409	95 °C for 2 min – 1 cycle	95 °C – 35 s	58 °C – 25 s	74 °C – 35 s	74 °C – for 2 min – 1 cycle	15 mcl
		33 cycle				

The temperature-time regimen of PCR was optimized for amplification of the studied polymorphic DNA sites

The primers were tested for lack of complementarity between the 3′-ends to prevent the formation of primer dimers and lack of internal secondary structures.

In this study, PCR of *PNPLA3* DNA sequences was performed on a thermal cycler (Tertsyk, DNA technology, Russia or GeneAmp ® 9700, Applied Biosystems, USA) using commercial test kit GenePak@ PCR Core (Isogen Laboratory) according to the manufacturer's protocol. For amplification of studied polymorphic DNA sites, the temperature-time regimes of PCR have been optimized (Table 2).

Detection of the PCR products was performed by horizontal electrophoresis in 2.5% agarose gel plate with the addition of ethidium bromide (an intercalating fluorescent DNA colorant) using standard tris-borate buffer at a field strength ~20 V/cm for 30 min. Ultraviolet energy, which was absorbed by the DNA in the range of 260 nm, was transmitted to the colorant, causing it to fluoresce in the orange-red range of the visible spectrum (590 nm) as an orange strip.

The results of the electrophoresis were assessed using transmitted ultraviolet light on transilluminator TFP-M/WL (Vilber Lourmat). The results were fixed using standard gel documenting system using software (ViTran-1, Biokom).

Determining the DNA nucleotide sequence. Nucleotide sequences of PCR fragments were detected using the Sanger method (Sanger and Coulson 1975) on an automatic DNA-sequencer (Genetic Analyzer 3130, Applied Biosystems, USA) using a sequencing kit (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, USA).

This is based on the use of four-color-labeled dideoxyribonucleotide terminators of chain elongation: G, A, T, and C (DyeDeoxy™ terminators, Applied Biosystems, USA). When replacing terminators on the standard deoxynucleotides in enzymatic sequencing, 3′ labeled products are produced that automatically analyzes by system. To each matrix was added 8 ml BigDye® Terminator v3.1 which consists of DyeDeoxy™ dNTPs and DNA polymerase AmpliTaq, 20 ng double-stranded DNA matrix, and 4 pmol each of the primers. The final volume of the reaction mix was adjusted to 20 μl. Sequencing reactions included initial denaturation at 96 °C for 1 min and 25 subsequent cycles that included denaturation at 96 °C for 10 s, primer hybridization at 50 °C for 5 s, and chain synthesis at 60 °C for 4 min. The reaction was performed on the GeneAmp® PCR System 9700 (Applied Biosystems, USA).

Reaction products were purified (BigDye XTerminator® Purification Kit, Applied Biosystems, USA). Samples were dissolved in 20 ml of formamide, mixed and denaturated at 95 °C for 2 min. Electrophoresis was performed on an automatic sequencer according to the manufacturer's protocol. The sequence was analyzed using software (Sequencing Analysis, Applied Biosystems, USA, and Chromas 1.55, Technelysium LTD, Australia).

Statistical analyses. Statistical analyses were performed using Statistica for Windows version 6.0. The quantitative data were not normally distributed as determined by the Kolmogorov-Smirnov criteria, so data are presented as median and interquartile range. Qualitative data is described using frequencies. The nonparametric data were analyzed using the Kruskal-Wallis test and the median test. To determine the significance of the differences between groups represented as an alternative variation, Fisher's exact test was used.

Prevalence of Obesity, Cardiovascular Risk Factors, and Cardiac Disease in the Study Population

In the population studied, 85.4% of patients were overweight; and 56.2% of patients had disorders of carbohydrate metabolism (i.e., glucose intolerance or type 2 diabetes mellitus). Hypertension was detected in 54.2% of patients with NAFLD, dyslipidemia in 50%, and coronary heart disease in 38.9%.

The Contribution of Genetic Polymorphism of rs 738409 *PNPLA3* Gene to the Development of NAFLD in Combination with Obesity and Cardiovascular Risk

Results of the PCR of the SNP rs738409 of the *PNPLA3* gene are shown in Figs. 1 and 2. Analysis of the frequency distribution of the rs738409 *PNPLA3* genotypes revealed that patients with NAFLD usually had the G allele ($\chi^2 = 14.10$;

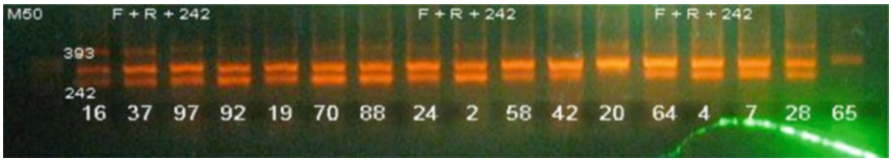

Fig. 1 PCR-reaction plate showing "stripes" specific for the G allele of rs738409 *PNPLA3*. Samples showing the G allele of the single nucleotide polymorphism rs738409 *PNPLA3* with control reaction (external primers annealing at 393 *p.n.*). The samples numbered 16, 37, 97, 92, 19, 70, 88, 24, 2, 58, 42, 64, 4, 7, and 28 are from carriers of the G allele. The specific band at 242 *p.n* is an interaction of a pair of primers: external forward and internal specific reverse. As a control reaction, an interaction of external primers was used (393 *p.n.*). Samples number 20 and 65 do not have allele G, being from mutants with genotype CC

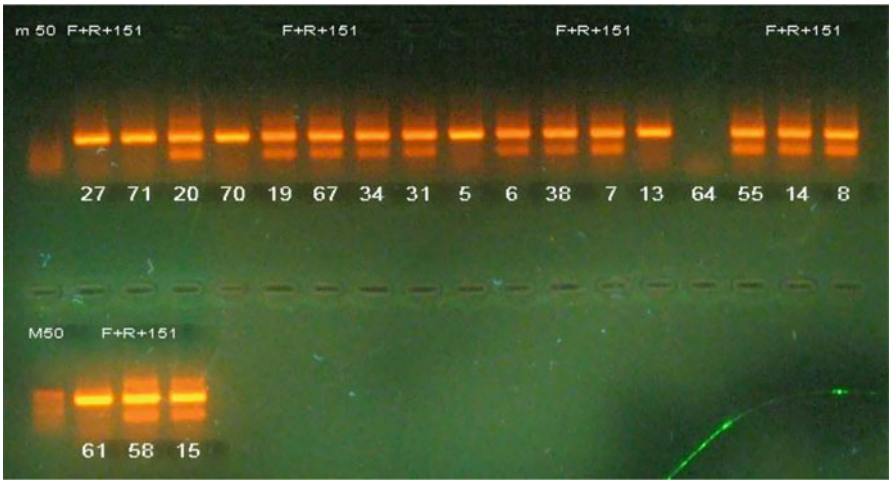

Fig. 2 PCR-reaction plate showing "stripes" specific for the C allele of rs 738409 PNPLA3. Samples showing the presence of the C allele of rs738409 *PNPLA3* with control reaction (external primers annealing at 393 *p.n.*). Samples 20, 19, 39, 67, 34, 31, 6, 38, 7, 55, 14, 8, 58, and 15 are carriers of the C allele (band of specificity at 151 *p.n.* as the result of primers pair interaction: external reverse and internal specific forward). External primer (393 *p.n.*) interaction was used as a control reaction. Samples 27, 71, 70, 5, 13, and 61 are not carriers of the C allele and therefore have the genotype GG

Table 3 Allele frequencies and genotypes of rs 738409 *PNPLA3* in healthy subjects and obese patients with NAFLD

Alleles and genotypes	without NAFLD, $n = 50$	With NAFLD, $n = 378$
Allele G	80.00%	92.15%
χ^2 14.10; $p = 0.0017$		
Allele C	70.00%	47.05%
χ^2 14.90; $p = 0.0011$		
GC	50.00%	41.17%
GG	30.00%	52.94%
CC	20.00%	5.88%
χ^2 22.30; $p = 0.0001$		

Comparing groups of patients with and without NAFLD demonstrated a significant predominance of the G allele and the GG genotype of rs 738409 *PNPLA3* in patients with NAFLD

$p = 0.0017$). Genotypes GG and GC were present in 52.94% and 41.17% of patients with NAFLD, respectively ($\chi^2 = 22.30$; $p = 0.0001$; Table 3).

Genotype also influenced the severity of hepatic steatosis ($\chi^2 = 23.93$; $p = 0.0008$; Table 4). The GG genotype results in more severe hepatic steatosis than the GC genotype (see Fig. 3). Thus, the G allele is dominant in hepatic steatosis (Fig. 4). This is confirmed by the data in Table 5 which also highlights the significant association ($p = 0.0005$) between the G allele and the degree of hepatic steatosis. The G allele is a marker of hepatic steatosis.

Table 4 Variation of the frequency of rs 738 409 *PNPLA3* genotypes with the degree of hepatic steatosis

	Genotypes rs 738409 gene PNPLA3		
Degree of hepatic steatosis	GC	GG	CC
1	48.39%	29.03%	22.58%
2	54.76%	45.24%	0%
3	38.10%	61.90%	0%
p = 0.0008. χ^2 23.93			

The prevalence of genotype GG of rs 738409 *PNPLA3* increased significantly as the degree of hepatic steatosis increases ($p = 0.0008$)

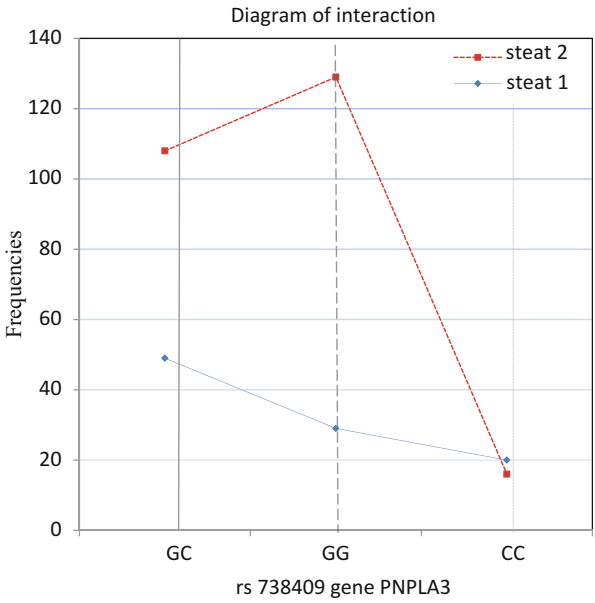

Fig. 3 Effect of polymorphism of rs738409 *PNPLA3* on the degree of hepatic steatosis. This graph demonstrates the effect of reducing the frequency of the CC genotype of rs 738409 *PNPLA3* on increasing stage of hepatic steatosis

The C allele did not significantly influence the development of hepatic steatosis ($p = 0.18$). To better understand the role of the C allele, see Fig. 5. The C allele was present more often in healthy subjects and was absent in some patients with NAFLD. The C allele is therefore not a marker of hepatic steatosis.

Analysis of the interaction between gender and the allele frequencies/genotypes of rs738409 *PNPLA3* in obese patients with NAFLD revealed significant differences. Genotype GG was more common in men, while allele C was more common in women. The differences were significant ($p = 0.0001$; $\chi^2 = 28.15$; Table 6). Obese men with NAFLD usually do not have the C allele (Fig. 6).

To detect relationships between *PNPLA3* and metabolic parameters, we analyzed serum lipid profile, markers of glycemic control, markers of liver damage, and adipose tissue hormone concentrations in patients with NAFLD depending on their rs738409 *PNPLA3* genotype (Table 7).

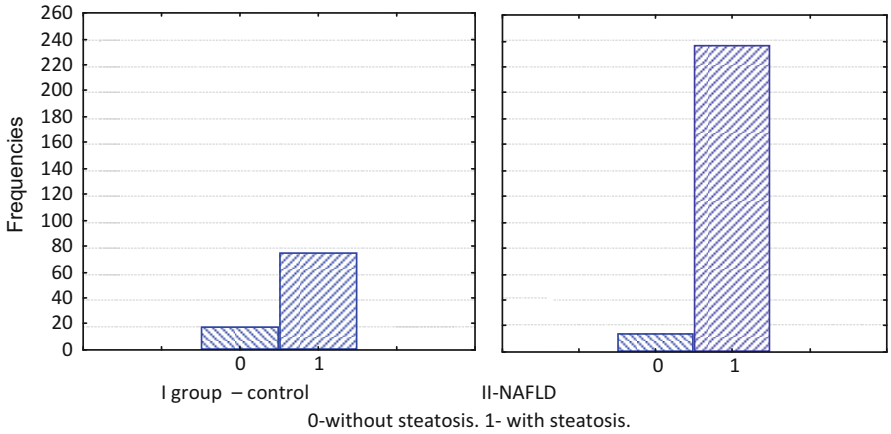

Fig. 4 The variation of the frequency of the G allele with degree of hepatic steatosis. The graph shows that the frequency of the G allele of rs 738409 *PNPLA3* increases as the degree of hepatic steatosis increases

Table 5 Variation of rs 738 409 *PNPLA3* G allele frequency with the degree of hepatic steatosis

Degree of hepatic steatosis	G allele frequency	
	G −	G +
1	22.58%	77.42%
2	0%	100%
3	0%	100%
$p = 0.0005$. χ^2 19.63		
Degree of hepatic steatosis	C allele frequency	
	C −	C +
1	29.03%	70.97%
2	45.24%	54.76%
3	61.90%	38.10%
$p = 0.18$. χ^2 7.98		

Analysis of the association of G and C alleles of rs 738409 *PNPLA3* with degree of hepatic steatosis demonstrated that the number of G allele carriers increased significantly as hepatic steatosis increased. The C allele was more common in patients with less severe hepatic steatosis

The parameters (Table 8) varied significantly with the genotypes of rs738409 *PNPLA3*. These serum analytes were more deranged in carriers of the G allele than those with the C allele. The results suggest that patients with NAFLD and obesity who have the G allele of rs738409 *PNPLA3* are at higher risk than those with the C allele of disease progression due to accumulation of fat in the liver.

Indicators of carbohydrate and lipid metabolism, indicators which characterize progression of NAFLD and obesity (DPI, TIMP, total fibrinogen), and parameters that damage to the liver (α2-, β-, γ-globulins, total bilirubin, direct and indirect bilirubin, ALT, AST) showed significant changes in patients with NAFLD who had the genotype GG. These changes were less severe in patients with NAFLD and the

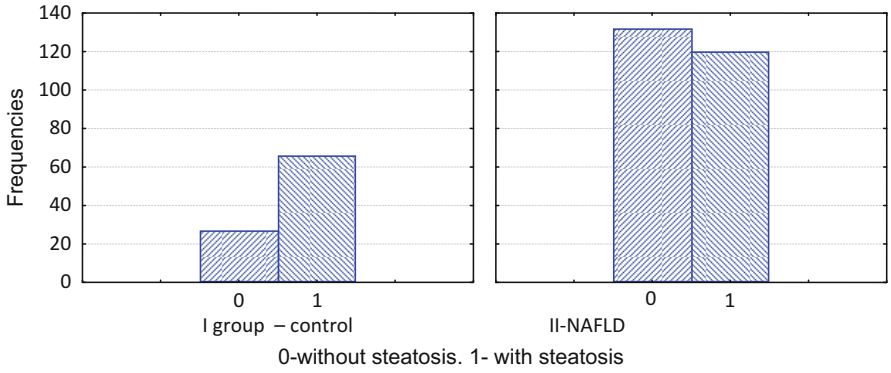

Fig. 5 The variation of the frequency of the C allele with degree of hepatic steatosis. The graph shows the frequency distribution of the C allele of rs 738409 *PNPLA3*. As the C allele is usually absent in patients with NAFLD, it probably has no effect on the incidence of the disease

Table 6 Gender distribution of the alleles and genotypes of rs 738409 *PNPLA3* in obese patients with NAFLD

Alleles and genotypes	Males, $n = 246$	Females, $n = 132$
Allele G	96.72%	90.24%
$P > 0.05$		
Allele C	40.98%	63.41%
$p = 0.026$. χ^2 4.93		
GC	37.70%	53.66%
GG	59.02%	36.59%
CC	3.28%	9.76%
$p = 0.0001$ χ^2 28.15		

There was no difference between male and female obese patients with NAFLD in the frequency of the G allele ($p > 0.05$). The C allele was more common in women. The GG genotype of rs 738409 *PNPLA3* was more common in men (59.02%), and the majority of obese women with NALFD had the GC genotype (53.66%, $p = 0.0001$)

GC genotype of SNP rs738409 *PNPLA3*. These differences as assessed by the Kruskal-Wallis test are highly significant (Table 9).

The data presented in Tables 8 and 9 indicate the involvement of the G allele both in the initiation and progression of NAFLD and obesity. Thus, our data demonstrate that patients who have the GG or GC genotypes of rs 738409 *PNPLA3* have a higher risk of developing NAFLD than carriers of the CC genotype.

Discussion

The *PNPLA3* gene encodes adiponutrin, a membrane-bound protein which although predominantly expressed in adipose tissue is also expressed in the liver. Data on the effect of rs738409 *PNPLA3* polymorphism on serum ALT and AST activities are

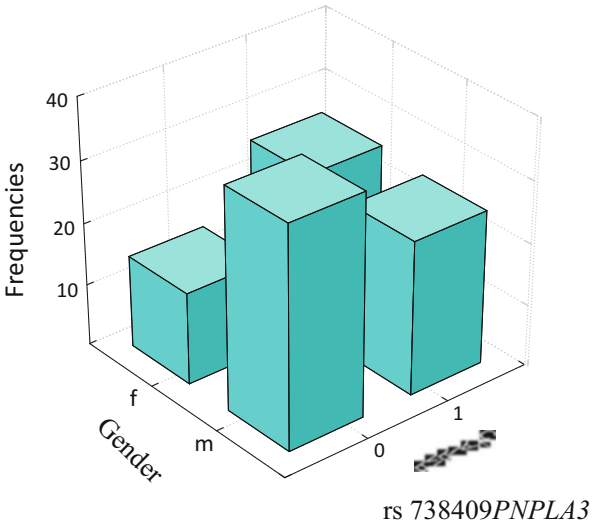

Fig. 6 The variation of the allele frequency of rs 738409 *PNPLA3* in male and female obese patients with NAFLD. This graph shows a lack of the allele frequency of rs 738409 *PNPLA3* in obese NAFLD males

contradictory. In Hispanics (Speliotes et al. 2010, 2011), Argentines, and Italians with NAFLD (Kollerits et al. 2009, 2010; Schweiger et al. 2009; Valenti et al. 2010a, b), the rs738409 G allele was associated with higher levels of serum ALT activity. In Fins (Kotronen et al. 2009a, b; Kantartzis et al. 2009; Speliotes et al. 2010) and Italians with obesity (Romeo et al. 2010a, b), the G allele is significantly associated with increased levels of AST. However, in African Americans and Germans (Wilson et al. 2006; Tian et al. 2010), rs738409 polymorphism is not associated with increased serum ALT or AST activities.

In our study which was conducted in Ukraine, the G allele of rs738409 was associated with increased levels of ALT and AST. This may be because the population that we studied were obese and had NAFLD. Previous studies have focused on patients who were either obese or had NAFLD. However, the data probably also varies between ethnic groups, at least in part, because the incidence of the rs738409 G allele varies between populations. Furthermore, several studies have shown that the activities of ALT and/or AST do not reflect the prevalence or severity of NAFLD (Mofrad et al. 2003).

Kollerits et al. (2009, 2010) reported that the rs738409 G allele was associated with low serum levels of total cholesterol and LDL cholesterol, but not HDL cholesterol and triglycerides. Speliotes et al. (2010, 2011) reported that the G allele is associated with a reduction in triglycerides in NAFLD. In our data, the *PNPLA3* rs738409 G allele was associated with reduced TG level in obese patients with NAFLD. This may be because TG production is reduced as a result of liver fibrosis in NAFLD.

Our data is consistent with previous data from experimental and clinical studies, indicating a relationship between rs738409 *PNPLA3* and fat accumulation in the liver. This suggests that I148M may affect lipid accumulation in hepatic steatosis. Thus, we believe that rs738409 *PNPLA3* polymorphism is involved in the development of liver steatosis and leads to NAFLD and obesity.

Table 7 The effect of genotype of rs738409 *PNPLA3* on adiposity, serum markers of liver disease and serum lipid profile in obese patients with NAFLD

Characteristic	Statistical indexes						
	Mean	Median	Minimum	Maximum	Lower quartile	Upper quartile	Standard deviation
GG genotype							
Indexes of adipose tissue							
BFM	33.37	34.30	22.40	44.60	29.20	37.40	5.15
VAT/SAT	0.18	0.20	0.02	0.39	0.09	0.27	0.10
LFC	10.21	10.20	3.20	19.30	8.20	13.30	3.65
Serum liver enzyme activities and serum lipid profile							
AST, U/l	0.48	0.42	0.10	1.78	0.24	0.60	0.35
ALT, U/l	0.81	0.64	0.14	2.70	0.47	1.00	0.58
GGT, U/l	81.52	68.50	0.70	510.00	38.00	107.00	82.08
ALP, nmol/l	1760.1	1780.0	0.65	2777.0	1580.0	1950.0	427.5
TC, mmol/l	6.61	6.41	3.93	7.97	5.25	7.53	1.10
TG, mmol/l	1.78	1.70	0.30	4.53	1.00	2.15	0.90
GC genotype							
Indexes of adipose tissue							
BFM	33.05	34.20	22.80	44.50	30.60	36.50	4.75
VAT/SAT	12.12	10.82	4.92	21.71	8.41	16.81	4.66
LFC	9.94	9.80	2.10	18.30	8.30	12.40	3.47
Serum liver enzyme activities and serum lipid profile							
AST, U/l	0.51	0.49	0.10	0.96	0.37	0.68	0.22
ALT, U/l	0.84	0.81	0.28	2.00	0.54	1.10	0.42
GGT, U/l	65.6	54.0	23.0	112.0	37.00	101.0	30.86
ALP, nmol/l	1904.0	1860.0	1440.0	2400.0	1758.0	2020.0	228.04
TC, mmol/l	5.68	5.88	0.98	7.20	5.06	6.46	1.06
TG, mmol/l	2.05	1.90	0.33	4.83	1.32	2.38	0.94
CC genotype							

(*continued*)

Table 7 (continued)

Characteristic	Statistical indexes						
	Mean	Median	Minimum	Maximum	Lower quartile	Upper quartile	Standard deviation
Indexes of adipose tissue							
BFM	32.78	32.40	28.50	37.60	31.50	34.30	2.81
VAT/SAT	0.22	0.23	0.07	0.34	0.21	0.26	0.08
LFC	6.48	6.55	3.60	7.80	7.60	6.80	1.17
Serum liver enzyme activities and serum lipid profile							
AST, U/l	0.61	0.51	0.36	1.20	0.47	0.58	0.28
ALT, U/l	0.75	0.61	0.20	1.76	0.54	0.79	0.49
GGT, U/l	76.75	79.50	63.00	85.00	69.00	84.50	9.07
ALP, nmol/l	1941.3	1857.5	1600.0	2450.0	1720.0	2162.5	320.3
TC, mmol/l	5.43	5.49	4.64	6.32	4.91	5.72	0.56
TG, mmol/l	1.63	1.50	0.79	3.28	0.82	1.90	0.86

Data on serum lipid profiles, serum liver enzyme activities (biochemical markers of liver disease), and indexes of adiposity are presented as mean (M), standard deviation (SD), median (Me), minimum, maximum, and quartiles (Q25; Q75). No significant differences were identified

Table 8 Statistical significance (p) of differences between the genotypes of rs738409 *PNPLA3* (χ^2 test)

Index	χ^2	The level of significance, p
LFC	8.36	0.0152
IMT index	5.77	0.0557
Resistin	11.23	0.0036
Hb_{1C}	15.7	0.0004
GGT	17.07	0.0002
Direct bilirubin	9.44	0.0089
Indirect bilirubin	6.39	0.0410
TC	17.14	0.0002

Comparison of the indicators presented in this table in patients with different genotypes of rs 738409 *PNPLA3* shows the effect of the G allele on progression of NAFLD

Table 9 Probability of dependence (p) of differences between the genotypes of rs738409 *PNPLA3* in various parameters (Kruskal-Wallis test)

Index	The level of significance
Indexes of carbohydrate metabolism	
Insulin	0.0294
C-peptide	0.0047
Indexes of lipid metabolism	
VLDL cholesterol	0.0004
TG	0.0001
Indexes of liver damage and protein-synthetic liver function	
ALT	0.0073
AST	0.0011
α2 – globulin	0.0000
γ-globulin	0.0539
Indexes of the liver fibrosis	
TIMP	0.0000
Fibrinogen	0.0055
Indexes of imaging diagnosis	
DPI	0.0054

There are the most significant changes in the indices that characterize the progression of NAFLD, the activity of inflammation observed in carriers of the GG genotype of rs 738409 *PNPLA3* according to Kruskal-Wallis test

Analysis of our data on the cardiovascular risk associated with the genotypes of obese patients with NAFLD revealed a significant contribution of rs 738409 *PNPLA3* polymorphism to cardiovascular risk ($p = 0.0258$; $\chi^2 = 11.06$; Table 10). Both the G allele ($p = 0.024$) and the C allele ($p = 0.05$) affect cardiovascular risk in obese patients with NAFLD (Table 11). The GG genotype influenced the cardiovascular risk most significantly. This observation is unlikely to be coincidental because PNPLA3 has a significant role in the regulation of serum lipid concentration.

In summary the results of our study of the SNP rs738409 of the adiponutrin gene (*PNPLA3*) in obese patients with NAFLD revealed that the G allele and GG

Table 10 Frequency distribution of genotypes of rs738409 *PNPLA3* with cardiovascular risk

Cardiovascular risk	Genotypes of rs738409 *PNPLA3*		
	GC	GG	CC
Low	57.14%	42.86%	0%
Moderate	46.67%	44.44%	8.89%
High	37.21%	58.14%	4.65%
$p = 0.0258$. criterion χ^2 11.06			

A significant proportion of patients with NAFLD and high cardiovascular risk had the GG genotype of rs 738409 *PNPLA3* ($p = 0.0258$)

Table 11 Frequency distribution of alleles of rs738409 *PNPLA3* with cardiovascular risk

Level of cardiovascular risk	Frequency C allele	
	C −	C +
Low	42.86%	57.14%
Moderate	44.44%	55.56%
High	58.14%	41.86%
$p = 0.05$. criterion χ^2 5.94		
Level of cardiovascular risk	Frequency G allele	
	G −	G +
Low	0%	100%
Moderate	8.89%	91.11%
High	4.65%	95.35%
$p = 0.024$. criterion χ^2 7.39		

Both the G and C alleles may be present in patients with high cardiovascular risk, but the G allele predominates ($p = 0.024$)

genotypes are associated with the development of liver steatosis. As it contributes significantly to cardiovascular risk in obese patients with NAFLD, determination of the presence of the G allele of rs738409 PNPLA3 may be useful to guide the use of primary prevention strategies.

Knowledge of the genetic predisposition to NAFLD and obesity and formation of cardiovascular risk in these patients allows risk stratification and should promote early administration of preventive and, if needed, therapeutic measures to limit disease progression. Furthermore, understanding of *PNPLA3* polymorphism could provide specific targets to prevent or treat obesity, NAFLD, or cardiovascular disease.

Potential Applications to Prognosis, Other Diseases, or Conditions

Mechanisms by which PNPLA3 variability may affect the content of triglycerides in the liver have been proposed, but the precise role of PNPLA3 I148M polymorphisms in the development of NAFLD is unclear. Association of SNP with the development of NAFLD and liver damage has been shown in several populations: Hispanics, Argentines, Italians adults and children with obesity, and Finns (Oliveira et al. 2011;

Kantartzis et al. 2007; Sookoian and Pirola 2011; Valenti et al. 2012; Anstee et al. 2011). Hotta et al. (2010) found a strong association between the G allele of rs738709 *PNPLA3* and NAFLD (especially NASH), increased activities of ALT and AST in plasma, reduced plasma triglycerides concentration, and more severe liver fibrosis (which is associated with n worse prognosis).

The results of our study show that carriers of the G allele of rs738409 *PNPLA3* have an increased risk of developing NAFLD. Compared with individuals with the homozygous genotype CC, carriers of CG heterozygous and GG homozygous genotypes have a significantly higher risk of NAFLD. The genotype may influence the progression of NAFLD and may also affect the prognosis. The high mortality rate of NAFLD is predominantly due to cardiovascular complications. "Fatty" liver may be an independent risk factor for cardiovascular events (Schindhelm et al. 2007).

Our data which demonstrates that the presence of NAFLD is strongly associated with increased risk of cardiovascular disease (OR = 3.208; 95% CI 1.4–2.1; $P < 0.001$) is consistent with previous reports (Targher et al. 2006). The prevalence of obesity, diabetes, and hypertension is high in patients with NAFLD. The effect of NAFLD on cardiovascular risk and progression of coronary artery disease is significant. Thus, to improve the outcome of patients with NAFLD, it is necessary to consider both hepatic steatosis and cardiovascular risk associated with *PNPLA3* polymorphisms alongside the traditional risk factors. This will increase the accuracy of prognostication and will optimize the management which should aim to limit the progression of hepatic steatosis and prevent cardiovascular complications.

Summary Points

Recent genome-wide association studies revealed that the genetic variation rs738409 (I148M) in PNPLA3 influences NAFLD and liver fat content.

The gene *PNPLA3* encodes a 481-aminoacid protein that contains a highly conserved patatin-like domain at the N terminus (PNPLA3 or adiponutrin).

Our data on the association of liver fat accumulation with the G allele of rs738409 *PNPLA3* is consistent with previous experimental and clinical studies indicating a link between rs738409 *PNPLA3* and liver fat content. This suggests that the I148M variant of PNPLA3 may affect lipid accumulation in fatty liver.

Increased liver fat content is a feature of metabolic disorders.

In patients with NAFLD, the G allele of rs738409 *PNPLA3* was significantly associated with increases in ALT ($P = 0.0073$), AST ($P = 0.0011$), TIMP-1 ($P = 0.0000$), TG ($P = 0.0001$), and VLDL ($P = 0.004$) levels even accounting for age, gender, and BMI.

Our results indicate that the rs738409 may be associated with liver fibrosis in NAFLD.

The GG genotype carrier of rs 738409 influences cardiovascular risk. Thus, we believe that PNPLA3 causes fatty infiltration of the liver and leads to the development of NAFLD and cardiovascular disease.

References

Anstee QM, Daly AK, Day CP. Genetics of alcoholic and non-alcoholic fatty liver disease. Semin Liver Dis. 2011;31:128–46.

Bellentani S, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. Dig Dis. 2010;28:155–61.

Farrell GC. PNPLeAse get the fats right: does lipogenesis or lipolysis cause NASH? Hepatology. 2010;52:818–21.

He S, McPhaul C, Li JZ, Garuti R, et al. A sequence variation (I148M) in PNPLA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis. J Biol Chem. 2010;285:6706–15.

Hooper AJ, Adams LA, Burnett JR. Genetic determinants of hepatic steatosis in man. Lipid Res. 2011;52:593–617.

Hotta K, Yoneda M, Hyogo H, Ochi H, Mizusawa S, Ueno T, Chayama K, Nakajima A, Nakao K, Sekine A. Association of the rs738409 polymorphism in PNPLA3 with liver damage and the development of nonalcoholic fatty liver disease. BMC Med Genet. 2010;11:172.

Huang Y, He S, Li JZ, Seo YK, et al. A feed-forward loop amplifies nutritional regulation of PNPLA3. Proc Natl Acad Sci U S A. 2010;107:7892–7.

Johansson LE, Lindblad U, Larsson CA, Rastamet L, et al. Polymorphisms in the adiponutrin gene are associated with increased insulin secretion and obesity. Eur J Endocrinol. 2008; 159:577–83.

Kakkos SK, Yarmenitis SD, Tsamandas AC, et al. Liver in obesity: relation to Doppler perfusion index measurement of the liver. Scand J Gastroenterol. 2000;35:976–80.

Kantartzis K, Fritsche A, Machicao F. Upstream transcription factor 1 gene polymorphisms are associated with high antilipolytic insulin sensitivity and show gene–gene interactions. J Mol Med. 2007;85:55–61.

Kantartzis K, Peter A, Machicao F, et al. Dissociation between fatty liver and insulin resistance in humans carrying a variant of the patatin–like phospholipase 3 gene. Diabetes. 2009;58:2616–23.

Kollerits B, Coassin S, Beckmann ND, et al. Genetic evidence for a role of adiponutrin in the metabolism of apolipoprotein B–containing lipoproteins. Hum Mol Genet. 2009;18:4669–76.

Kollerits B, Coassin S, Kiechl S, Hunt SC, et al. A common variant in the adiponutrin gene influences liver enzyme. J Med Genet. 2010;4:116–9.

Kotronen A, Peltonen M, Hakkarainen A, et al. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. Gastroenterology. 2009a;137:865–72.

Kotronen A, Johansson LE, Johansson LM, Roos C, et al. Common variant in *PNPLA3* which encodes adiponutrin is associated with liver fat content in humans. Diabetologia. 2009b;52:1056–60.

Krawczyk M, Mullenbach R, Weber SN, et al. Genome wide association studies and genetic risk assessment of liver diseases. Gastroenterol Hepatol. 2010;7:669–81.

Mofrad P, Contos MJ, Haque M, et al. Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. Hepatology. 2003;37:1286–92.

Mohammadinia AR, Bakhtavar K, Ebrahimi–Daryani N, Habibollahi P, et al. Correlation of hepatic vein Doppler waveform and hepatic artery resistance index with the severity of nonalcoholic fatty liver disease. J Clin Ultrasound. 2010;38:346–52.

Oliveira CS, Fernando M, Giuffrida A, et al. ADIPOQ and adiponectin: the common ground of hyperglycemia and coronary artery disease? Arq Bras Endocrinol Metab. 2011;55 (7):446–54.

Palmer CN, Maglio C, Pirazzi C, et al. Paradoxical lower serum triglyceride levels and higher type 2 diabetes mellitus susceptibility in obese individuals with the PNPLA3 148M variant. PLoS ONE. 2012. http://doi:10.1371/journal.pone.0039362.

Park BJ, Kim YJ, Kim DH, Kim W, et al. Visceral adipose tissue area is an independent risk factor for hepatic steatosis. J Gastroenterol Hepatol. 2008;23:900–7.

Petit JM, Guiu B, Masson D, Duvillard L, et al. Specifically PNPLA3-mediated accumulation of liver fat in obese patients with type 2 diabetes. J Clin Endocrinol Metab. 2010;95:E430–6.

Pingitore P, Pirazzi C, Mancina RM, et al. Recombinant PNPLA3 protein shows triglyceride hydrolase activity and its I148M mutation results in loss of function. Biochim Biophys Acta. 2013;574–80.

Pirazzi C, Adiels M, Burza MA, et al. Patatin-like phospholipase domain containing 3 (PNPLA3) I148M (rs738409) affects hepatic VLDL secretion in humans and in vitro. J Hepatol. 2012;6:1276–82.

Rae-Whitcombe SM, Kennedy D, Voyles M, Thompson MP. Regulation of the promoter region of the human adiponutrin/PNPLA3 gene by glucose and insulin. Biochem Biophys Res Commun. 2010;402:767–72.

Ratziu V, Voiculescu M, Poynard Ratziu TV, et al. Touching some firm ground in the epidemiology of NASH. J Hepatol. 2012;56:23–5.

Romeo S, Kozlitina J, Xing C, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet. 2008;40:1461–5.

Romeo S, Huang-Doran I, Baroni MG, Kotronen A, et al. Unravelling the pathogenesis of fatty liver disease: patatin-like phospholipase domain-containing 3 protein. Curr Opin Lipidol. 2010a;21:247–52.

Romeo S, Sentinelli F, Dash S, et al. Morbid obesity exposes the association between PNPLA3 I148M (rs738409) and indices of hepatic injury in individuals of European descent. Int J Obes (Lond). 2010b;34:190–4.

Ruhanen H, Perttila JD, Holtta-Vuori MD, et al. PNPLA3 mediates hepatocyte triacylglycerol remodelling. J Lipid Res. 2014;55:739–46.

Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol. 1975;94:441–8.

Santoro N, Kursawe R, D'Adamo E, Dykas DJ, et al. A common variant in the patatin-like phospholipase 3 gene (PNPLA3) is associated with fatty liver disease in obese children and adolescents. Hepatology. 2010;52:1281–90.

Schindhelm RK, Dekker JM, Nijpels G. Alanine aminotransferase predicts coronary heart disease events: a 10–year follow–up of the Hoorn Study. Atherosclerosis. 2007;191:391–6.

Schweiger M, Lass A, Zimmermann R, Eichmann TO, et al. Neutral lipid storage disease: genetic disorders caused by mutations in adipose triglyceride lipase/PNPLA2 or CGI-58/ABHD5. Am J Physiol Endocrinol Metab. 2009;297:E289–96.

Sookoian S, Pirola CJ. Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease. Hepatology. 2011;53:1883–94.

Sookoian S, Castaño GO, Burgueño AL, Gianotti TF, Rosselli MS, Pirola CJ. A nonsynonymous gene variant in the adiponutrin gene is associated with nonalcoholic fatty liver disease severity. J Lipid Res. 2009;50:2111–6.

Speliotes EK. Genetics of common obesity and nonalcoholic fatty liver disease. Gastroenterology. 2009;136:1492–5.

Speliotes EK, Butler JL, Palmer CD, et al. PNPLA3 variants specifically confer increased risk for histologic nonalcoholic fatty liver disease but not metabolic disease. Hepatology. 2010;52:904–12.

Speliotes EK, Yerges-Armstrong LM, Wu J. Genome-wide association analysis identifies variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits. PLoS Genet. 2011. http://www.ncbi.nlm.nih.gov/pubmed/21423719192.

Stein JH. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima–Media Thickness Task Force Endorsed by the Society for Vascular Medicine. J Am Soc Echocardiogr. 2008;21:94–111.

Targher G, Bertolini L, Padovani R, Poli F, Scala L, Tessari R, Zenari L, Falezza G. Increased prevalence of cardiovascular disease in type 2 diabetic patients with non-alcoholic fatty liver disease. Diabet Med. 2006;23:403–9.

Tian C, Stokowski RP, Kershenobich D, Ballinger DG, et al. Variant in PNPLA3 is associated with alcoholic liver disease. Nat Genet. 2010;42:21–3.

Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology. 2010;52:1836–46.
Valenti L, Al-Serri A, Daly AK, Galmozzi E, et al. Homozygosity for the patatin–like phospholipase–3/adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease. Hepatology. 2010a;51:1209–17.
Valenti L, Alisi A, Galmozzi E, Bartuli A, et al. I148M patatin-like phospholipase domain-containing 3 gene variant and severity of pediatric nonalcoholic fatty liver disease. Hepatology. 2010b;52:1274–80.
Valenti L, Rametta R, Ruscica M, Dongiovanni P, et al. The I148M PNPLA3 polymorphism influences serum adiponectin in patients with fatty liver and healthy controls. BMC Gastroenterol. 2012;12:111.
Wang CW, Lin HY, Shin SJ, et al. The PNPLA3 I148M polymorphism is associated with insulin resistance and nonalcoholic fatty liver disease in a normoglycaemic population. Liver Int. 2011;31:1326–31.
Williams CD, Stenger J, Asike MI, et al. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle–aged population utilizing ultrasound and liver biopsy: a prospective study. Gastroenterology. 2011;140:124–31.
Wilson PA, Gardner SD, Lambie NM, Commans SA, et al. Characterization of the human patatin-like phospholipase family. J Lipid Res. 2006;47:1940–9.
Wong VW. Non-alcoholic fatty liver disease in Asia – a story of growth. J Gastroenterol Hepatol. 2013;28:18–23.
Younossi ZM, Stepanova M, Rafiq N, Makhlouf H, Younoszai Z, Agrawal R, Goodman Z. Pathologic criteria for nonalcoholic steatohepatitis: interprotocol agreement and ability to predict liver-related mortality. Hepatology. 2011;53:1874–82.
Yuan X, Waterworth D, Perry JR, Lim N, et al. Population-based genomewide association studies reveal six loci influencing plasma levels of liver enzymes. Am J Hum Genet. 2008;83:520–8.
Zhan YT, Su HY, An W. Glycosyltransferases and non-alcoholic fatty liver disease. World J Gastroenterol. 2016;22:2483–93.

Histological Biomarkers of Nonalcoholic Fatty Liver Disease

33

Giuseppe Derosa and Pamela Maffioli

Contents

G. Derosa (✉)
Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for the Study of Endocrine-Metabolic Pathophysiology and Clinical Research, University of Pavia, Pavia, Italy

Laboratory of Molecular Medicine, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
e-mail: giuseppe.derosa@unipv.it

P. Maffioli (✉)
Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

PhD School in Experimental Medicine, University of Pavia, Pavia, Italy
e-mail: pamelamaffioli@hotmail.it

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_23

Abstract

Nonalcoholic fatty liver disease (NAFLD) is an important complication of the metabolic syndrome, which is becoming an increasingly common cause of chronic liver disease. Nonalcoholic fatty liver disease is characterized by a wide spectrum of conditions and covers a histological spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis, and cirrhosis.

Liver biopsy remains the gold standard for characterizing liver histology in patients with NAFLD; however, it is expensive and carries some morbidity and very rare mortality risk, so it should be performed in those who would benefit the most from diagnostic, therapeutic guidance, and prognostic perspectives.

In this regard, the aim of this chapter will be to examine histological biomarkers of NAFLD in order to give readers a guide about diagnosis and follow-up of this kind of disease.

Keywords

Cirrhosis • Fat accumulation • Fibrosis • Nonalcoholic fatty liver disease • Steatohepatitis

List of Abbreviations

HSC	Hepatic stellate cell
NAFL	Nonalcoholic fatty liver
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor-α

Key Facts of Nonalcoholic Fatty Liver Disease

- Simple steatosis without fibrosis or inflammation has a benign clinical course in most cases without excess mortality.
- Nonalcoholic steatohepatitis, instead, may have a more progressive course that can lead to cirrhosis in 10–15% of patients, affecting survival.
- Noninvasive methods, in particular ultrasonography, have been proposed to identify and grade NAFL. However, the distinction between pure NAFL and NASH can only be made histologically.
- The knowledge of histological differences between NAFL and NASH is very important to an accurate differentiation between the two conditions.

Definition of Words and Terms

Biomarker	The term refers to a measurable indicator of some biological state or condition that can be used for diagnosis or follow-up of a particular disease.
Fibrosis	The term refers to an overly exuberant wound healing in response to a chronic injury, in which excessive connective tissue builds up in the liver, resulting in disruption of normal hepatic architecture.
Hepatic steatosis	The term refers to death of hepatic parenchyma which may involve single cell or multicell in piece-meal, focal, periacinar, midzonal, periportal, or paracentral locations.
Nonalcoholic fatty liver	The term refers to a condition characterized by the presence of hepatic steatosis with no evidence of hepatocellular injury in the form of ballooning of the hepatocytes.
Nonalcoholic steatohepatitis	The term refers to a condition characterized by the presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning), with or without fibrosis.

Definition of Nonalcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is an important complication of the metabolic syndrome, which is becoming an increasingly common cause of chronic liver disease. Nonalcoholic fatty liver disease is characterized by a wide spectrum of conditions and covers a histological spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis, and cirrhosis.

According to guidelines (Chalasani et al. 2012), to diagnose NAFLD, two conditions are required:

- Evidence of hepatic steatosis, either by imaging or by histology
- No causes for secondary hepatic fat accumulation such as significant alcohol consumption, use of steatogenic medication, or hereditary disorders

Regarding causes of secondary hepatic steatosis, we can identify causes of macrovesicular and microvesicular steatosis. Causes of macrovesicular steatosis include excessive alcohol consumption, hepatitis C (genotype 3), Wilson's disease,

lipodystrophy, starvation, parenteral nutrition, abetalipoproteinemia, and some drugs such as amiodarone, methotrexate, tamoxifen, and corticosteroids. On the other side, causes of microvesicular steatosis include Reye's syndrome, some drugs such as valproate and anti-retroviral medicines, acute fatty liver of pregnancy, HELLP syndrome, and inborn errors of metabolism (including LCAT deficiency, cholesteryl ester storage disease, Wolman disease).

Histologically, NAFLD can be categorized into:

- Nonalcoholic fatty liver (NAFL) characterized by the presence of hepatic steatosis with no evidence of hepatocellular injury in the form of ballooning of the hepatocytes
- Nonalcoholic steatohepatitis (NASH) characterized by the presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning), with or without fibrosis

Simple steatosis without fibrosis or inflammation has a benign clinical course in most cases without excess mortality. NASH, instead, may have a more progressive course that can lead to cirrhosis in 10–15% of patients, affecting survival (Ekstedt et al. 2006). It develops in subjects who are not heavy alcohol consumers and have negative tests for viral and autoimmune liver diseases (Matteoni et al. 1999; Brunt 2001). Recently, NAFLD has been linked to insulin resistance and type 2 diabetes mellitus and metabolic syndrome (Cortez-Pinto et al. 1999; Marchesini et al. 2001).

Liver biopsy remains the gold standard for characterizing liver histology in patients with NAFLD; however, it is expensive and carries some morbidity and very rare mortality risk, so it should be performed in those who would benefit the most from diagnostic, therapeutic guidance, and prognostic perspectives.

In this regard, the aim of this chapter will be to examine histological biomarkers of NAFLD in order to give readers a guide about diagnosis and follow-up of this kind of disease.

Mechanisms Responsible for Nonalcoholic Fatty Liver Disease

As already said above, the presence and severity of NAFLD are closely related to risk factors for insulin resistance and the metabolic syndrome (Reid 2001). The first step in NAFLD development is fat accumulation in hepatocytes, and this process is closely associated with metabolic derangements related to central obesity and insulin resistance. An increased delivery of free fatty acids to the liver is combined with impaired fatty acid metabolism in hepatocytes, leading to a net accumulation of triglyceride within the liver (Hübscher 2006). Increased hepatocellular expression of the microsomal cytochrome P450 2E1 (CYP2E1) seems to have an important role in mediating this process (Schattenberg et al. 2005), which leads to a potential vicious cycle where the metabolic syndrome causes fatty change in the liver and vice versa. The progression from simple steatosis to more complex conditions is due to peroxidation of lipid

accumulated within steatotic hepatocytes by induction of hepatic CYP2E128 and mitochondrial dysfunction leading to the formation of reactive oxygen species (ROS). Lipid peroxidation products trigger immune responses that contribute to disease progression (Albano et al. 2005). Furthermore, metabolic syndrome is characterized by increased levels of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, which may be released directly from adipocytes in visceral fat, and decreased levels of anti-inflammatory cytokines such as adiponectin. These cytokines seem to play an active role in the developing of the process; this is confirmed by the fact that a significantly reduced intrahepatic expression of adiponectin and its receptors has been observed in biopsies showing NASH compared with simple steatosis (Kaser et al. 2005). Moreover, interactions between lipid-laden hepatocytes, Kupffer cells, inflammatory cells, and hepatic stellate cells contribute to the development of fibrosis and cirrhosis. Fibrosis consists of an overly exuberant wound healing in response to a chronic injury, in which excessive connective tissue builds up in the liver; when this process results in disruption of normal hepatic architecture, it results in cirrhosis, which can lead to portal hypertension.

Factors involved in the regulation of this process include activation of interleukin-10 and transforming growth factor-β (Bugianesi et al. 2005). Lipid released from damaged hepatocytes may also result in mechanical and inflammatory cell-mediated occlusion of hepatic venules, again leading to parenchymal collapse and fibrosis (Wanless and Shiota 2004).

Histological Markers of Nonalcoholic Fatty Liver Disease

As already said above, liver biopsy remains the gold standard for characterizing liver histology in patients with NAFLD. The main histological characteristic of NAFLD is the accumulation of fat in the form of triglycerides within hepatocytes; the presence of >5% steatotic hepatocytes in a liver tissue section is now accepted as the minimum criterion for the histological diagnosis of NAFLD (Neuschwander-Tetri et al. 2003). Histological changes occurring in NAFLD typically predominantly affect the liver parenchyma, where they are mainly present in perivenular regions (acinar zone 3), even if in severe cases it can extend to a panacinar distribution. Changes observed in the liver are often reversible, especially in the early stages of the disease; later, when NAFLD progresses to fibrosis and cirrhosis, the damage becomes permanent.

We can histologically classify steatosis into:

- Microvesicular steatosis: characterized by the presence of numerous small vesicles of fat that do not displace the nucleus
- Macrovesicular steatosis: characterized by hepatocytes with a single large intracytoplasmic fat droplet or smaller well-defined droplets displacing the nucleus to the cell periphery

In NAFLD, fatty change is predominantly macrovesicular, even if mixed steatosis, with macrovesicular and microvesicular steatosis, might also occur. Brunt et al. (1999) proposed a score to classify the severity of fatty change, evaluating the proportion of hepatocytes containing fat droplets; in particular, we classify steatosis into:

- Mild steatosis when the proportion of hepatocytes containing fat droplets involve <33% of hepatocytes
- Moderate steatosis when the proportion of hepatocytes containing fat droplets involve a proportion >33% and <66% of hepatocytes
- Severe steatosis when the proportion of hepatocytes containing fat droplets involve a proportion >66% of hepatocytes

In NAFLD, in addition to steatosis, foci of lobular inflammation, mild portal inflammation, and lipogranulomas may be seen.

Histological Markers of Nonalcoholic Steatohepatitis

Nonalcoholic steatohepatitis is defined by the presence of steatosis accompanied by various elements (Table 1):

- Hepatocyte injury.
- Lobular inflammation, typically localized in acinar zone 3.
- Fibrosis can be present, but it is not necessary to diagnose NASH.

Hepatocellular Injury

Hepatocellular injury in NASH can take the form of ballooning (the most common one), apoptosis, or lytic necrosis.

Table 1 Histological characteristic of NASH

Hepatocellular injury
Ballooning
Apoptosis
Mallory's hyaline
Giant mitochondria
Inflammation
Neutrophil polymorphs
Other cells (e.g., T lymphocytes, macrophages)
Fibrosis
Perisinusoidal
Pericellular

Ballooned hepatocytes are enlarged, with swollen, rarefied, pale cytoplasm, and, usually, show a large, hyperchromatic nucleus, often with a prominent nucleolus. Loss of the normal hepatocyte keratins (Hossain et al. 2009), as detected by immunostaining, might help in the objective identification of ballooned hepatocytes (Lackner et al. 2008).

Death of hepatocytes may occur by apoptosis or lytic necrosis, even if lytic necrosis following ballooning degeneration is most common in alcoholic steatohepatitis, not in NASH.

Apoptotic (acidophil) bodies, instead, are typical of NASH and can easily be identified on routine stains, but are further highlighted by immunohistochemistry for keratin 18 fragments that has been recently proposed as a biomarker of NASH (Feldstein et al. 2009). The number of acidophil bodies per mm^2 of liver tissue (acidophil body index) has been proposed to serve as a complementary histological feature when diagnosis of NASH is uncertain.

Hepatocyte ballooning is typically associated with formation of Mallory's hyaline. Mallory bodies in NAFLD may be difficult to detect in routinely stained sections because they are usually small and poorly formed (Nonomura et al. 2005). For this reason, immunohistochemical techniques have been proposed to demonstrate antigens associated with Mallory's hyaline, such as ubiquitin, p62, and cytokeratins 8 and 18 (Banner et al. 2000; Zatloukal et al. 2002). Mallory bodies have been correlated with increased necroinflammatory activity (Matteoni et al. 1999) and with a higher incidence of cirrhosis. Their presence is not required for the histological identification of NASH, even if the presence of Mallory bodies strengthens the diagnosis.

Megamitochondria, more common in alcoholic liver disease, have been identified also in NASH (Le et al. 2004). Morphologically, the main characteristic is the development of mitochondrial swelling with formation of crystalline inclusions within the mitochondrial matrix. The crystalline inclusions are true crystals, although their composition remains uncertain (Sternlieb and Berger 1969). Megamitochondria with crystalline inclusions have been reported in 5–15% of hepatocytes in patients with NASH; crystalline inclusions typically appear in close association with swollen mitochondria, which may be rounded or elongated. The crystals occur as long parallel strands, which often deform the shape of the mitochondrion. Each single crystalline strand is approximately 10 nm in diameter, and typically, there is 20 nm between strands (Caldwell et al. 1999). It has been reported that mitochondrial defects in the form of loss of crystalline and paracrystalline inclusions were present in patients with NASH, but not in those with NAFLD, supporting the hypothesis that mitochondrial abnormalities play a role in the pathogenesis of progressive liver injury in NAFLD.

Inflammation

Regarding inflammation, it is usually mild lobular inflammation and comprises a mixed inflammatory cell infiltrate, composed of neutrophils, lymphocytes (mainly

CD3+ T cells), some eosinophils, and also macrophages/Kupffer cells. Foci of chronic lobular inflammation, consisting mainly of lymphocytes, are occasionally seen. Scattered lobular microgranulomas (Kupffer cell aggregates) and lipogranulomas are common (Takahashi et al. 2014).

Portal chronic mononuclear cell inflammation in adult NASH is not uncommon and is usually mild; in untreated NAFLD patients, increased portal inflammation has been proposed as a marker of severe disease (Brunt et al. 2009).

Fibrosis

The parenchymal fibrosis that occurs in fatty liver disease typically has a perisinusoidal and/or pericellular distribution. In adult, NASH usually starts in acinar zone 3 and has a characteristic "chicken wire" pattern due to deposition of collagen and other extracellular matrix fibers along the sinusoids of zone 3 and around the hepatocytes. Sinusoidal collagen formation in NASH is mainly due to hepatic stellate cell (HSC) activation and the portal fibroblasts, which are activated by soluble mediators produced by activated hepatic resident cells, most importantly Kupffer cells, and by inflammatory cells infiltrating the liver during chronic hepatic diseases. The HSC activation score, as measured by alpha-smooth muscle actin immunohistochemistry, was shown to predict progression of fibrosis in NAFLD (Feldstein et al. 2005). In advanced disease, bridging fibrosis and cirrhosis might develop. Cirrhosis is a pathological condition characterized by architectural distortion of the liver, including diffuse parenchymal nodularity, fibrosis, and vascular changes. Cirrhosis can be divided into:

- Macronodular cirrhosis characterized by large nodules, >3 mm, surrounded by fibrous septa
- Micronodular cirrhosis characterized by small nodules, <3 mm, surrounded by fibrous septa
- Mixed cirrhosis characterized by both small and large nodules surrounded by fibrous septa

Nonalcoholic steatohepatitis-related cirrhosis is most commonly macronodular or mixed (Brunt et al. 2009).

Grading of Nonalcoholic Fatty Liver Disease

Noninvasive methods such as computed tomography (CT), magnetic resonance imaging (MRI), and sonography have been proposed to identify and grade NAFL; the most diffuse method is ultrasonography, because it is cost effective and widely available, even if it is limited by interobserver and intra-observer variability (Strauss et al. 2007).

For an approximate estimation of hepatic steatosis, hepatic parenchyma can be compared to kidney parenchyma during ultrasound examination: in normal conditions, the liver and renal cortex are of a similar echogenicity; in steatosis, instead, the renal cortex appears hypoechoic compared to the liver parenchyma. The brighter is hepatic parenchyma compared to the kidney one, the higher is the steatosis degree. A better grading of severity of hepatic steatosis is possible with an ultrasound score, according to this score:

- Level 0 was defined as a normal hepatic echo pattern.
- Level 1 was defined as a slight increase in echo pattern with normal visualization of vessels and the diaphragm.
- Level 2 was defined as a moderate increase in echogenicity with reduced visibility of portal veins and the diaphragm.
- Level 3 was defined as a pronounced increase in hepatic echo pattern with poor visibility of intrahepatic vessels and posterior right lobe of the liver.

This score derives from the evaluation of different aspects of the liver during ultrasound examination, which considers liver echotexture, echo penetration and visibility of the diaphragm, and clarity of liver blood vessel structures (Chan et al. 2004).

Although fatty change can be reliably diagnosed by noninvasive methods, the distinction between pure fatty change (NAFL) and NASH can only be made histologically. In this regard, the American Association for the Study of Liver Diseases has produced a consensus document in which a number of essential and nonessential features of steatohepatitis have been identified (Table 2). Summarizing, the necessary criteria to diagnose NASH include:

- Steatosis, mainly macrovesicular and in zone 3
- Mixed, mild lobular inflammation polymorphs as well as mononuclear cells
- Hepatocyte ballooning, most apparent near steatotic cells

Regarding fibrosis, instead, several histological scorings have been proposed to classify the severity of fibrosis.

The score proposed by Brunt et al. (1999) to assess fibrosis degree considers the presence and severity of steatosis, ballooning, and inflammation. On this basis, four stages of fibrosis are considered (Table 3):

- Stage 1 is characterized by initial involvement of perisinusoidal spaces in zone 3.
- Stage 2 involves the subsequent development of portal/periportal fibrosis.
- Stage 3 includes bridging fibrosis.
- Stage 4 includes cirrhosis.

This score has been lately replaced by a score proposed by the Clinical Research Network (CRN) (Kleiner et al. 2005), which derives from the summation of

Table 2 Histological definition of NASH

Necessary components to diagnose NASH
Steatosis, macro > micro, mainly zone 3
Mixed, mild lobular inflammation polymorphs as well as mononuclear cells
Hepatocyte ballooning, most apparent near steatotic cells
Usually present, but not necessary for diagnosis of NASH
Perisinusoidal fibrosis (zone 3)
Glycogenated nuclei (zone 1)
Lipogranulomas (usually small)
Occasional acidophil bodies or periodic acid-Schiff-stained Kupffer cells

Table 3 Grading activity and staging fibrosis in NASH according to Brunt et al. (1999)

	Steatosis	Ballooning	Inflammation (counted in 20 × fields)	
			L – lobular (stages 0–3)	P – portal (stages 0–3)
			[0, absent; 1, <2; 2, 2–4; 3, >4 foci]	[0, absent; 1, mild; 2, moderate; 3, severe]
Grade 1	1–2	Minimal (zone 3)	1–2	0–1
Grade 2	2–3	Present (zone 3)	1–2	0–1
Grade 3	2–3	Marked (mainly zone 3)	3	2

Table 4 Grading activity and staging fibrosis in NASH according to Kleiner et al. (2005)

Steatosis (S) grade	Lobular (L) inflammation counted in 20 × fields	Hepatocyte ballooning (B)
0, <5%	0, none	0, none
1, 5–33%	1, <2	1, few ballooned cells
2, 34–66%	2, 2–4	2, many ballooned cells
3, >66%	3, >4	

individual scores for steatosis (S), lobular (L) inflammation, and hepatocellular ballooning (B) and ranges from 0 to 8 (Table 4).

In particular, stages of fibrosis are classified as:

- Stage 0: absence of fibrosis
- Stage 1a: mild fibrosis, with delicate zone 3 perisinusoidal fibrosis
- Stage 1b: moderate fibrosis, with dense zone 3 perisinusoidal fibrosis
- Stage 1c: portal/periportal fibrosis only
- Stage 2: zone 3 perisinusoidal fibrosis and portal/periportal fibrosis
- Stage 3: bridging fibrosis
- Stage 4: cirrhosis, probable or definite

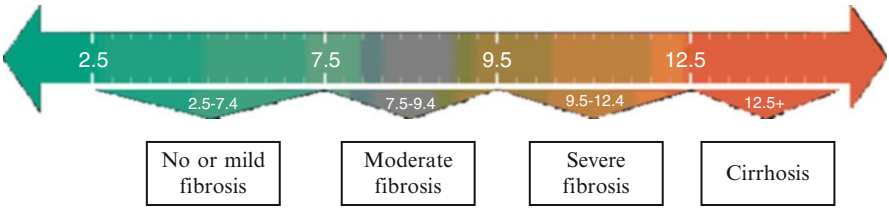

Fig. 1 FibroScan score and fibrosis degree

Hepatic fibrosis can be quantified also in a noninvasive way, throughout assessment of stiffness, using an ultrasound-based technology introduced in the latest years. This technique called transient ultrasound elastography or FibroScan measures the stiffness of the hepatic parenchyma using both ultrasound (5 MHz) and low-frequency (50 Hz) elastic waves produced by a specialized ultrasound vibrator applied to the body wall and coupled with 1D ultrasound imaging that measures the propagation speed of a wave using a pulse-echo ultrasound. Since fibrotic tissue is harder than healthy liver tissue, the shear wave measurement provides immediate quantitative assessment of the degree of stiffness. FibroScan was reported to be a reliable method for the diagnosis of significant fibrosis (AUC = 0.84), severe fibrosis (AUC = 0.89), and cirrhosis (AUC = 0.94) accompanying various liver diseases including hepatitis B and C, alcoholic liver disease, and NAFLD (Ziol et al. 2005; Friedrich-Rust et al. 2008). However, previously reported papers showed that FibroScan accuracy in assessing lower degrees of liver fibrosis is not as reliable compared to diagnosing advanced fibrosis and cirrhosis (Ziol et al. 2005).

Stiffness assessed by FibroScan is expressed in kPa, using a score between 2.5 and 75 kPa. Between 90% and 95% of healthy people without liver disease will have a liver scarring measurement less than 7.0 kPa; patients with chronic hepatitis C and a liver stiffness more than 14 kPa have approximately a 90% probability of having cirrhosis, while patients with liver stiffness more than 7 kPa have around an 85% probability of at least significant fibrosis (Fig. 1).

Potential Applications to Prognosis, Other Diseases, or Conditions

Noninvasive methods such as computed tomography (CT), magnetic resonance imaging (MRI), and in particular ultrasonography have been proposed to identify and grade NAFL. However, the distinction between pure NAFL and NASH can only be made histologically. The knowledge of histological differences between NAFL and NASH is very important to an accurate differentiation between the two conditions. NASH, in fact, is the most dangerous condition because it may have a more progressive course that can lead to cirrhosis in 10–15% of patients, affecting survival. An early identification of NASH can help to early treat it, because, as we have already said above, at first structural hepatic changes are reversible.

Summary Points

- This chapter focuses on histological biomarkers of nonalcoholic fatty liver disease.
- Nonalcoholic fatty liver is characterized by the presence of hepatic steatosis with no evidence of hepatocellular injury in the form of ballooning of the hepatocytes.
- Nonalcoholic steatohepatitis (NASH) is characterized by the presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning), with or without fibrosis.
- The knowledge of histological differences between NAFL and NASH is very important to an accurate differentiation between the two conditions.

References

Albano E, Mottaran E, Vidali M, Reale E, Saksena S, Occhino G, Burt AD, Day CP. Immune response towards lipid peroxidation products as a predictor of progression of non-alcoholic fatty liver disease to advanced fibrosis. Gut. 2005;54:987–93.

Banner BF, Savas L, Zivny J, Tortorelli K, Bonkovsky HL. Ubiquitin as a marker of cell injury in nonalcoholic steatohepatitis. Am J Clin Pathol. 2000;114:860–6.

Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol. 1999;94:2467–74.

Brunt EM. Nonalcoholic steatohepatitis: definition and pathology. Semin Liver Dis. 2001; 21(1):3–16.

Brunt EM, Kleiner DE, Wilson LA, Unalp A, Behling CE, Lavine JE, et al. Portal chronic inflammation in nonalcoholic fatty liver disease (NAFLD): a histologic marker of advanced NAFLD-Clinicopathologic correlations from the nonalcoholic steatohepatitis clinical research network. Hepatology. 2009;49:809–20.

Bugianesi E, McCullough AJ, Marchesini G. Insulin resistance: a metabolic pathway to chronic liver disease. Hepatology. 2005;42:987–1000.

Caldwell SH, Swerdlow RH, Khan EM, Iezzoni JC, Hespenheide EE, Parks JK, Parker Jr WD. Mitochondrial abnormalities in non-alcoholic steatohepatitis. J Hepatol. 1999;31:430–4.

Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. Hepatology. 2012;55(6):2005–23.

Chan DF, Li AM, Chu WC, Chan MH, Wong EM, Liu EK, et al. Hepatic steatosis in obese Chinese children. Int J Obes Relat Metab Disord. 2004;28(10):1257–63.

Ekstedt M, Franzén LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, Kechagias S. Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology. 2006;44(4):865–73.

Feldstein AE, Papouchado BG, Angulo P, Sanderson S, Adams L, Gores GJ. Hepatic stellate cells and fibrosis progression in patients with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol. 2005;3:384–9.

Feldstein AE, Wieckowska A, Lopez AR, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. Hepatology. 2009;50:1072–8.

Friedrich-Rust M, Ong MF, Martens S, Sarrazin C, Bojunga J, Zeuzem S, et al. Performance of transient elastography for the staging of liver fibrosis: a meta-analysis. Gastroenterology. 2008;134(4):960–74.

Hossain N, Afendy A, Stepanova M, Nader F, Srishord M, Rafiq N, Goodman Z, Younossi Z. Independent predictors of fibrosis in patients with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol. 2009;7:1224–9.

Hübscher SG. Histological assessment of non-alcoholic fatty liver disease. Histopathology. 2006;49:450–65.

Kaser S, Moschen A, Cayon A, Kaser A, Crespo J, Pons-Romero F, Ebenbichler CF, Patsch JR, Tilg H. Adiponectin and its receptors in non-alcoholic steatohepatitis. Gut. 2005;54:117–21.

Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41:1313–21.

Lackner C, Gogg-Kamerer M, Zatloukal K, Stumptner C, Brunt EM, Denk H. Ballooned hepatocytes in steatohepatitis: the value of keratin immunohistochemistry for diagnosis. J Hepatol. 2008;48:821–8.

Le TH, Caldwell SH, Redick JA, Sheppard BL, Davis CA, Arseneau KO, Iezzoni JC, Hespenheide EE, Al-Osaimi A, Peterson TC. The zonal distribution of megamitochondria with crystalline inclusions in nonalcoholic steatohepatitis. Hepatology. 2004;39:1423–9.

Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, McCullough AJ, Natale S, Forlani G, Melchionda N. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes. 2001;50(8):1844–50.

Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology. 1999;116:1413–9.

Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. Hepatology. 2003;37:1202–19.

Nonomura A, Enomoto Y, Takeda M, Tamura T, Kasai T, Yoshikawa T, et al. Clinical and pathological features of non-alcoholic steatohepatitis. Hepatol Res. 2005;33:116–21.

Reid AE. Nonalcoholic steatohepatitis. Gastroenterology. 2001;121:710–23.

Schattenberg JM, Wang Y, Singh R, Rigoli RM, Czaja MJ. Hepatocyte CYP2E1 overexpression and steatohepatitis lead to impaired hepatic insulin signaling. J Biol Chem. 2005;280:9887–94.

Sternlieb I, Berger JE. Optical diffraction studies of crystalline structures in electron micrographs. J Cell Biol. 1969;43:448–55.

Strauss S, Gavish E, Gottlieb P, Katsnelson L. Interobserver and intraobserver variability in the sonographic assessment of fatty liver. AJR. 2007;189(6):W320–3.

Takahashi Y, Fukusato T. Histopathology of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J Gastroenterol. 2014;20(42):15539–48.

Wanless IR, Shiota K. The pathogenesis of nonalcoholic steatohepatitis and other fatty liver diseases: a four-step model including the role of lipid release and hepatic venular obstruction in the progression to cirrhosis. Semin Liver Dis. 2004;24:99–106.

Zatloukal K, Stumptner C, Fuchsbichler A, et al. p62 Is a common component of cytoplasmic inclusions in protein aggregation diseases. Am J Pathol. 2002;160:255–63.

Ziol M, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, et al. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. Hepatology. 2005;41(1):48–54.

Vascular Cell Adhesion Molecule-1 (VCAM-1) Expression in Liver Disease

34

Giuseppe Derosa and Pamela Maffioli

Contents

G. Derosa (✉)
Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for the Study of Endocrine-Metabolic Pathophysiology and Clinical Research, University of Pavia, Pavia, Italy

Laboratory of Molecular Medicine, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
e-mail: giuseppe.derosa@unipv.it

P. Maffioli (✉)
Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

PhD School in Experimental Medicine, University of Pavia, Pavia, Italy
e-mail: pamelamaffioli@hotmail.it

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_24

Abstract

Cell adhesion molecules belong to a family called glycoproteins. They are involved in binding with other cells or with the extracellular matrix in the process called cell adhesion. In particular, vascular cell adhesion molecule-1 (VCAM-1) can bind to leucocyte integrin very late antigen-4 (VLA-4) to recruit leucocytes to sites of inflammation. Thus, VCAM-1 stimulates adhesion of lymphocyte and monocytes to the surface of the vascular endothelium.

There is evidence that soluble VCAM-1 concentration is increased in patients with liver disease, in comparison to control subjects, and, for this reason, it could be used as a biomarker to early identify liver disease. At this regard, the aim of this chapter will be to examine the role of VCAM-1 expression in liver disease.

Keywords

Cellular adhesion molecules • Inflammation • Hepatocarcinoma • Hepatitis • Liver disease

List of Abbreviations

ICAMs	Intercellular adhesion molecules
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
NAFLD	Nonalcoholic fatty liver disease
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4

Key Facts of Vascular Cell Adhesion Molecule-1 Expression in Liver Disease

- VCAM-1 stimulates adhesion of lymphocyte and monocytes to the surface of the vascular endothelium.
- Soluble VCAM-1 concentration is increased in patients with liver disease, in comparison to control subjects.
- Overexpression of VCAM-1 or VLA-4 may lead to several diseases, such as acute or chronic hepatitis and metastasis.
- Quantifying the affinity of VCAM-1/VLA-4 interaction can be of primary importance in devising biomarkers to better understand its role in diseases as well as in developing the therapeutic drugs and strategies.

Definitions of Words and Terms

Cell adhesion molecules	The term refers to a family of proteins located on the cell surface involved in binding with other cells or with the extracellular matrix in the process called cell adhesion.
Endothelium	The term refers to a type of epithelium that lines the interior surface of blood vessels and lymphatic vessels, forming an interface between circulating blood or lymph in the lumen and the rest of the vessel wall.
Leukocytes	The term refers to a family of cells of the immune system that are involved in protecting the body against both infectious disease and foreign invaders.
Metastasis	The term refers to a process by which cancer spreads from the place at which it first arose as a primary tumor to distant locations in the body.

Cell Adhesion Molecules

Cell adhesion molecules belong to a family of chemicals called glycoproteins. They are involved in binding with other cells or with the extracellular matrix (ECM) in the process called cell adhesion. Cell adhesion molecules are located at the cell surface and form different types of complexes and junctions to join:

- Cells to cells
- Cells to extracellular matrix
- Extracellular matrix to the cell cytoskeleton

Cell adhesion molecules have different functions, including:

- The adhesion of cells to one another to provide organized tissue structure
- The transmission of extracellular cues and signals across the cell membrane
- The migration of cells through the regulation of cell adhesion molecules

Extracellular matrix and cell adhesion molecules are involved in a large range of disorders and diseases; in some of these, adhesion is increased and in some decreased.

Actually, four main families of cell adhesion molecules have been discovered, with different functions.

- Cadherins: they depend on the presence of calcium ions to function; they are involved in cell to cell junctions. These molecules are transmembrane glycoproteins and link the cytoskeleton of one cell to the cytoskeleton of another.

- Integrins: they are involved in cell to matrix junctions; they mediate cell-matrix interactions.
- Immunoglobulins: they include vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecules (ICAMs). They have a role in inflammation, in immune responses, and in intracellular signaling events.
- Selectins: they bind to cell-surface carbohydrate and are involved with inflammation response mechanisms.

All these proteins are typically transmembrane receptors and are composed of three domains: an intracellular domain that interacts with the cytoskeleton, a transmembrane domain, and an extracellular domain that interacts either with other cellular adhesion molecules of the same kind (homophilic binding) or with other cellular adhesion molecules or the extracellular matrix (heterophilic binding).

Immunoglobulins Family

Intercellular Adhesion Molecules

As written above, immunoglobulin superfamily includes ICAMs and VCAM-1. They are important in inflammation, in immune responses, and in intracellular signaling events. The ICAM family includes five members, designated ICAM-1 to ICAM-5. They are known to bind to leukocyte integrins CD11/CD18 during inflammation and in immune responses. In addition, ICAMs may be found in soluble forms in human plasma, due to activation and proteolysis mechanisms at cell surfaces.

Vascular Cell Adhesion Molecule-1

Vascular cell adhesion molecule-1 or CD106 is a 110 kDa transmembrane glycoprotein member of the immunoglobulin gene superfamily (Osborn et al. 1989) (Fig. 1). It was first described as a cytokine-inducible endothelial adhesion molecule. It can bind to leukocyte integrin very late antigen-4 (VLA-4) to recruit leucocytes to sites of inflammation. Thus, VCAM-1 stimulates adhesion of lymphocyte and monocytes to the surface of the vascular endothelium. In addition, eosinophils and basophils, but not neutrophils, can bind to endothelial cells via VCAM-1/VLA-4 interaction. This adhesion molecule is expressed primarily on endothelial cells; however, other cell types, both vascular and nonvascular cells, are also capable of expressing VCAM-1. The predominant form of VCAM-1 in vivo has an N-terminal extracellular region comprising seven Ig-like domains. A conserved integrin-binding motif has been identified in domains 1 and 4, variants of which are present in the N-terminal domain of all members of the integrin-binding subgroup of the immunoglobulin superfamily. The structure of a VLA-4-binding fragment comprising the first two domains of VCAM-1 has been determined to 1.8 A resolution.

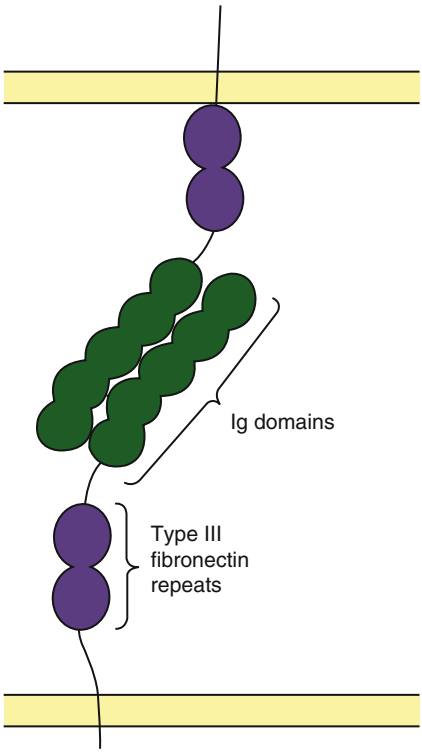

Fig. 1 Immunoglobulins family structure (Adapted from: Fokunang and Rastall 2003)

The integrin-binding motif is exposed and forms the N-terminal region of the loop between beta-strands C and D of domain 1. VCAM-1 domains 1 and 2 are structurally similar to ICAM-1 and ICAM-2.

Vascular Cell Adhesion Molecule-1 Function

As said above, VCAM-1 is expressed on a variety of vascular and nonvascular cells, where its primary function is to mediate intercellular adhesion. On vascular endothelium, expression is inducible by cytokine activation in a variety of inflammatory conditions and mediates leukocyte recruitment from blood into tissues. In particular, VCAM-1 promotes the adhesion of lymphocytes, monocytes, eosinophils, and basophils (Carlos et al. 1990; Rice et al. 1991) via its ligand, VLA-4. Although the interaction of VCAM-1 with VLA-4 is essential for immunity, overexpression of any of them or both may lead to several diseases pathologies such as multiple sclerosis (De Andres et al. 2012), rheumatoid arthritis (Takeuchi et al. 2002), allogeneic graft rejection, delayed-type hypersensitivity reactions, and tumor metastasis (Klemke et al. 2007). Thus quantifying the affinity of VCAM-1/VLA-4 interaction can be of primary importance in devising biomarkers to better understand its role in

diseases as well as in developing the therapeutic drugs and strategies. Interestingly, certain melanoma cells can use VCAM-1 to adhere to the endothelium, and VCAM-1 may participate in monocyte recruitment to atherosclerotic sites. The existence of soluble forms of VCAM-1 has already been reported in literature (Pigott et al. 1992), and its measurement may permit ready monitoring of inflammatory diseases or immunotherapy and be of help in clarifying immunopathogenetic issues (Simpson and Hayes 1995). Moreover, soluble VCAM-1 concentration is increased in patients with liver disease, in comparison to control subjects (Pirisi et al. 1996).

At this regard, the aim of this chapter will be to examine the role of VCAM-1 expression in liver disease so that it could be used as a biomarker to early identify liver disease.

Vascular Adhesion Molecules in Acute and Chronic Liver Inflammation

Liver contains a large resident and migratory population of lymphocytes and macrophages that provide immune surveillance against foreign antigens. This population can be rapidly expanded in response to infection or injury by recruiting leucocytes from the circulation, a process that is dependent on the ability of lymphocytes to recognize, bind to, and migrate across the endothelial cells that line the hepatic vasculature. The complex nature of the liver vasculature means that there are several points at which lymphocytes can interact with endothelium and be recruited into different anatomical compartments. The normal liver contains a large number of lymphocytes that include not only specialized NK and NKT cells, but also CD4 and CD8 T cells. As already said above, soluble VCAM-1 concentration is increased in patients with liver disease, in comparison to control subjects (Pirisi et al. 1996). Considering that the expression of VCAM-l mediates the adhesion of mononuclear blood cells, but not neutrophils, to the vascular endothelium (Shimizu et al. 1992), it would be plausible to expect an increase concentration of soluble VCAM-l mainly in those liver diseases characterized by a mononuclear infiltrate.

It has been reported that soluble VCAM-1 levels were significantly higher in patients with chronic hepatitis, compared to patients with acute hepatitis or compared to controls. Immunological staining showed that VCAM-1 was mainly expressed on the surface of sinusoidal endothelium and was not expressed on the hepatic parenchyma. Moreover, VLA-4 was detected on the surface of mononuclear inflammatory cells and Kupffer cells around hepatocytes in patients with acute hepatitis (Haruta et al. 1999). Given these observations, we can assume that acute viral infection stimulates immune response and cytokines release including TNF-α, and IL-1β. They stimulate the expression of VCAM-1; activated T cells expressing VLA-4 can infiltrate sinusoids and adhere to sinusoidal lining cells of liver throughout the VCAM-1/VLA-4 mechanism (Fig. 2). VCAM-1 was not detected on hepatocytes, so it does not directly contribute to hepatocytes damage, ICAMs does. Vascular adhesion molecule-1 was reported to increase with the progression of

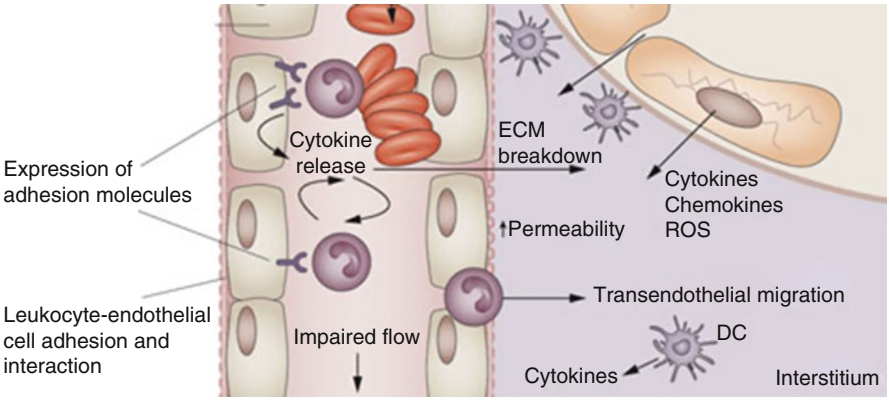

Fig. 2 Vascular cell adhesion molecule-1 expression (Adapted from: Sharfuddin and Molitoris 2011)

liver disease from acute hepatitis to chronic hepatitis to liver cirrhosis. Given that chronic hepatitis occurs when effector lymphocytes are recruited to the liver from blood and retained in tissue to interact with target cells, such as hepatocytes or bile ducts, and given that VCAM-1 supports leukocyte adhesion by binding α4β1 integrins, this mechanism is critical for the recruitment of monocytes and lymphocytes during inflammation (Afford et al. 2014).

While the severity of liver disease matters, soluble VCAM-l does not seem to be influenced by the etiology, toxic or viral, of the disease (Volpes et al. 1992). The highest levels occur in patients with acute hepatitis and in patients with advanced chronic liver disease, with lower levels measured in patients with only mild impairment of liver function. In particular, soluble VCAM-1 has been found elevated in patients with alcoholic cirrhosis, in comparison to patients with alcoholic hepatitis and steatosis (Adams et al. 1994). We have already said above that pro-inflammatory cytokines, such as TNF-α and IL-1β, are well-known inducers of vascular adhesion molecules, including VCAM-1. In addition, recent studies suggest that other cytokines, such as interleukin-4 (IL-4), may exhibit similar effects in various vascular cells (Blease et al. 1998). This finding has suggested that in patients with cirrhosis, local cytokines release might be shifted toward a prevalent IL-4 response, resulting in selective induction of VCAM-l, leading in turn to the predominantly mononuclear infiltrate observed in the cirrhotic liver.

Vascular cell adhesion molecule-1 may also have an important role in the progression of nonalcoholic fatty liver disease (NAFLD) and establishment of chronic liver disease. There are reports that hepatic expression of VCAM-1 is increased in human NAFLD in association with markedly elevated serum levels of soluble VCAM-1 when compared with those in controls matched for age, sex, and metabolic phenotype (Weston et al. 2015). Authors provided evidence that VCAM-1 promotes the progression of steatohepatitis, because VCAM-1-deficient mice (Aoc3-/-) and WT animals treated with a neutralizing antibody were protected

from the development of severe steatohepatitis and onset of fibrosis in three murine models of steatohepatitis and a carbon tetrachloride (CCl4) model of fibrosis.

The soluble forms of VCAM-l in patients with liver disease share a strict correlation with markers of cholestasis and of a reduced functioning hepatic mass. This raises the possibility that its concentration in serum might increase mainly because of an impairment of the excretory functions of the patients (Lim et al. 1994). Preliminary evidence seems to suggest that soluble VCAM-l is neither chemotactic nor proadhesive (Adams et al. 1994). It is conceivable that, in fact, soluble VCAM-l might compete for the same ligand recognized by the membrane-bound form, operating a negative feedback on the ability of leukocytes to reach the site of inflammation. If this was true, high soluble VCAM-1 levels might contribute to the impairment of immune surveillance observed in patients with advanced chronic liver disease.

Vascular Cell Adhesion Molecule-1 Expression in Hepatocellular Carcinoma

Most patients with hepatocellular carcinoma (HCC) who have undergone complete tumor resection developed subsequent recurrence of tumor. A follow-up study of patients who underwent liver resection for HCC found that more than 50% would develop recurrent cancer 5 years after the operation, even in those with small HCC (tumor smaller than 3 cm) and liver transplantation. The median duration of recurrence was 24 months (Cha et al. 2003). Hemming et al. (2001) noted that the overall survival rates of 112 patients with nonfibrolamellar HCC who underwent a liver transplant from 1985 to 2000 were 78%, 63%, and 57% at 1, 3, and 5 years, respectively. Patients infected with hepatitis B virus had a worser 5-year survival rate than those who had not (43% vs. 64%). Bai et al. (2007) observed that there is an effect of pro-inflammatory cytokines on the tumor growth, and VCAM-1 is implied. These authors measured some dominant pro-inflammatory cytokines, including TNF-α, IL-1β, interleukin-6 (IL-6), and VCAM-1 in the liver tissues of the mice after operation and tried to find out some correlations between these inflammatory cytokines and postoperative metastasis. The results showed that the expression of TNF-α mRNA in liver tissues increased gradually at 4 h and peaked at 96 h after partial hepatectomy. The highest expression level of IL-1 mRNA and IL-6 occurred at 72 h and 48 h after partial hepatectomy, respectively, then rapidly declined to normal level. The expression of VCAM-1 mRNA was almost undetectable in normal liver tissues, but significantly increased and peaked at 4 h and 72 h after partial hepatectomy. The up-regulated increased expression of VCAM-1 occurred at a different time from that of TNF-α, which indicated that the increased expression of VCAM-1 was not up-regulated by TNF-α. Cytokines and cell adhesion molecules were elevated especially in the initial 72 h after partial hepatectomy, which was the pivotal moment of liver cancer metastasis. Therefore, the elevated pro-inflammatory cytokines and cell adhesion molecules occurring in the initial 72 h after partial hepatectomy might be involved in early intrahepatic metastasis or recurrences. Pro-inflammatory cytokines IL-1β, IL-6, TNF-α, ICAMs, and VCAM-1 produced

by Kupffer cells, stellate cells, and endothelial cells in the remaining liver tissues, partially explained the cause of early intrahepatic recurrences after the radical resection of HCC.

Vascular Cell Adhesion Molecule-1 Expression and Metastasis

Metastasis is a major clinical problem and results in a poor prognosis for most cancers. The metastatic pathway describes the process by which cancer cells give rise to a metastatic lesion in a new tissue or organ. It consists of interconnecting steps all of which must be successfully completed to result in a metastasis. The adhesion of circulating cancer cells to capillary endothelial is a critical step in the initiation of metastasis. The liver is a major site of metastasis for some of the most common human malignancies, carcinomas of the gastrointestinal tract in particular. Liver metastases are frequently inoperable and are associated with poor prognosis (Boring et al. 1994). Metastatic tumor cells entering the liver trigger a pro-inflammatory response involving Kupffer cell-mediated release of TNF-α and the up-regulation of vascular endothelial cell adhesion receptors, such as E-selectin. Recent studies showed that several types of tumor-infiltrating lymphocytes are associated with improved disease outcome for various human cancers. The adhesive function of VCAM-1 is, in fact, used by cancer cells to enhance metastatic implantation and spread (Rice and Bevilacqua 1989). Under normal physiological conditions, E-selectin and VCAM-1 expression on vascular endothelial cells is low. In response to cytokines such as IL-1β and TNF-α, E-selectin expression is induced through activation of the nuclear factor-B and Raf/MEK/MAPK pathways, and this, in turn, can lead to up-regulation of VCAM-1 and ICAM-1 expression (Kim et al. 2001). In particular, IL-1β and TNF-α are known to potentiate the metastasis of VLA-4-expressing mouse B16 melanoma (B16M) cells in lung tissue by a mechanism which involves the up-regulation of VCAM-1 expression on hepatic sinusoidal endothelium cells (Okahara et al. 1994). In particular, melanoma cells release a prometastatic substance that increases TNF-α production in hepatic sinusoidal endothelium. TNF-α, in turn, stimulates IL-1β release, and mature IL-1β stimulates IL-18 release. Mature IL-18 induces VCAM-1, which facilitates the adhesion of melanoma cells to hepatic sinusoidal endothelium (Vidal-Vanaclocha et al. 1996).

Potential Applications to Prognosis, Other Diseases, or Conditions

All things considered, although the interaction of VCAM-1 with VLA-4 is essential for immunity, overexpression of any of them or both may lead to several diseases. Quantifying the affinity of VCAM-1/VLA-4 interaction can be of primary importance in devising biomarkers to better understand its role in diseases as well as in developing the therapeutic drugs and strategies. Also, the measurements of soluble

forms of VCAM-1 may permit ready monitoring of inflammatory diseases or immunotherapy and be of help in clarifying immunopathogenetic issues.

Summary Points

- VCAM-1 stimulates adhesion of lymphocyte and monocytes to the surface of the vascular endothelium.
- Soluble VCAM-1 concentration is increased in patients with liver disease, in comparison to control subjects.
- In this chapter, we examined the role of VCAM-1 expression in liver disease.
- Overexpression of VCAM-1 or VLA-4 may lead to several diseases, such as acute or chronic hepatitis and metastasis.
- Quantifying the affinity of VCAM-1/VLA-4 interaction can be of primary importance in devising biomarkers to better understand its role in diseases as well as in developing the therapeutic drugs and strategies.

References

Adams DH, Burra P, Hubscher SG, Elias E, Newman W. Endothelial activation and circulating vascular adhesion molecules in alcoholic liver disease. Hepatology. 1994;19:588–94.

Afford SC, Humphreys EH, Reid DT, Russell CL, Banz VM, Oo Y, Vo T, Jenne C, Adams DH, Eksteen B. Vascular cell adhesion molecule 1 expression by biliary epithelium promotes persistence of inflammation by inhibiting effector T-cell apoptosis. Hepatology. 2014; 59(5):1932–43.

Bai L, Mao GP, Cao CP. Effects of inflammatory cytokines on the recurrence of liver cancer after an apparently curative operation. J Dig Dis. 2007;8(3):154–9.

Blease K, Seybold J, Adcock IM, Hellewell PG, Burke-Gaffney A. Interleukin-4 and lipopolysaccharide synergize to induce vascular cell adhesion molecule-1 expression in human lung microvascular endothelial cells. Am J Respir Cell Mol Biol. 1998;18(5):620–30.

Boring CC, Squires TS, Tong T, Montgomery S. Cancer statistics. CA Cancer J Clin. 1994;44:7–26.

Carlos TM, Schwartz BR, Kovach NL, Yee E, Rosa M, Osborn L, Chi-Rosso G, Newman B, Lobb R, Harlan JM. Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine activated cultured human endothelial cells. Blood. 1990;76:965–70.

Cha C, Fong Y, Jarnagin WR, Blumgart LH, DeMatteo RP. Predictors and patterns of recurrence after resection of hepatocellular carcinoma. J Am Coll Surg. 2003;197:753–8.

De Andres C, Teijeiro R, Alonso B, Sánchez-Madrid F, Martínez ML, Guzmán de Villoria J, Fernández-Cruz E, Sánchez-Ramón S. Long-term decrease in VLA-4 expression and functional impairment of dendritic cells during natalizumab therapy in patients with multiple sclerosis. PLoS One. 2012;7(4), e34103.

Fokunang CN, Rastall RA. Phytohaemagglutinins in membrane signalling, biomedical and genetic engineering research. Biotechnology. 2003;2(2):162–77.

Haruta I, Tokushige K, Komatsu T, Ikeda I, Yamauchi K, Hayashi N. Clinical implication of vascular cell adhesion molecule-1 and very late activation antigen-4 interaction, and matrix metalloproteinase-2 production in patients with liver disease. Can J Gastroenterol. 1999; 13(9):721–7.

Hemming AW, Cattral MS, Reed AI, Van Der Werf WJ, Greig PD, Howard RJ. Liver transplantation for hepatocellular carcinoma. Ann Surg. 2001;233:652–9.

Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. J Biol Chem. 2001;276:7614–20.

Klemke M, Weschenfelder T, Konstandin MH, Samstag Y. High affinity interaction of integrin α4β1 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) enhances migration of human melanoma cells across activated endothelial cell layers. J Cell Physiol. 2007;212:368–74.

Lim AG, Jazrawi RP, Ahmed HA, Levy JH, Zuin M, Douds AC, Maxwell JD, Northfield TC. Soluble intercellular adhesion molecule-1 in primary biliary cirrhosis: relationship with disease stage, immune activity and cholestasis. Hepatology. 1994;20(4 Pt 1):882–8.

Okahara H, Yagita H, Miyake K, Okumura K. Involvement of very late activation antigen 4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) in tumor necrosis factor alpha enhancement of experimental metastasis. Cancer Res. 1994;54(12):3233–6.

Osborn L, Hession C, Tizard R, Vassallo C, Luhowskyj S, Chi-Rosso G, Lobb R. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell. 1989;59:1203–11.

Pigott R, Dillon LP, Hemingway IH, Gearing AJ. Soluble forms of E-selectin, ICAM-1 and VCAM-1 are present in the supernatants of cytokine activated cultured endothelial cells. Biochem Biophys Res Commun. 1992;187(2):584–9.

Pirisi M, Fabris C, Falleti E, Soardo G, Toniutto P, Vitulli D, Gonano F, Bartoli E. Serum soluble vascular-cell adhesion molecule-1 (VCAM-1) in patients with acute and chronic liver diseases. Dis Markers. 1996;13(1):11–7.

Rice GE, Bevilacqua MP. An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science. 1989;246:1303–6.

Rice GE, Munro JM, Corless C, Bevilacqua MP. Vascular and nonvascular expression of INCAM-110. A target for mononuclear leukocyte adhesion in normal and inflamed human tissues. Am J Pathol. 1991;138(2):385–93.

Sharfuddin AA, Molitoris BA. Pathophysiology of ischemic acute kidney injury. Nat Rev Nephrol. 2011;7(4):189–200.

Shimizu Y, van Seventer GA, Ennis E, Newman W, Horgan KJ, Shaw S. Crosslinking of the T cell-specific accessory molecules CD7 and CD28 modulates T cell adhesion. J Exp Med. 1992; 175(2):577–82.

Simpson KJ, Hayes PC. Soluble adhesion molecules in immune mediated liver disease. Gut. 1995;36(6):806–8.

Takeuchi E, Tanaka T, Umemoto E, Tomita T, Shi K, Takahi K, Suzuki R, Ochi T, Miyasaka M. VLA-4-dependent and -independent pathways in cell contact-induced proinflammatory cytokine production by synovial nurse-like cells from rheumatoid arthritis patients. Arthritis Res. 2002;4(6):R10.

Vidal-Vanaclocha F, Alvarez A, Asumendi A, Urcelay B, Tonino P, Dinarello CA. Interleukin 1 (IL-1)-dependent melanoma hepatic metastasis in vivo; increased endothelial adherence by IL-1-induced mannose receptors and growth factor production in vitro. J Natl Cancer Inst. 1996;88(3–4):198–205.

Volpes R, Van Den Oord JJ, Desmet VJ. Vascular adhesion molecules in acute and chronic liver inflammation. Hepatology. 1992;15(2):269–75.

Weston CJ, Shepherd EL, Claridge LC, Rantakari P, Curbishley SM, Tomlinson JW, Hubscher SG, Reynolds GM, Aalto K, Anstee QM, Jalkanen S, Salmi M, Smith DJ, Day CP, Adams DH. Vascular adhesion protein-1 promotes liver inflammation and drives hepatic fibrosis. J Clin Invest. 2015;125(2):501–20.

Immunohistochemistry for Viral Hepatitis: Methods and Applications

35

Cihan Yurdaydin

Contents

Abstract

Immunohistochemical methods have been used for the diagnosis and management of chronic viral hepatitis. Today their use in clinical practice is more limited. This is due to improvement in diagnostic methods, less need and desire to perform a liver biopsy, and advances in assessment of liver disease severity by noninvasive means. Antibodies developed for immunohistochemistry (IHC) have applications far beyond IHC and are applicable to various methods including ELISA, immunofluorescence, immunoprecipitation, flow cytometry, immunocytochemistry, and Western blotting. Such methods have contributed to our understanding of the biology and function of several viral proteins. However, IHC staining may still have a role for the diagnosis and management of some viral hepatitis forms such as HDV and CMV infections. This holds true especially in the posttransplant setting, where IHC is a useful adjunct for the diagnosis and

C. Yurdaydin (✉)
Department of Gastroenterology, University of Ankara Medical School, Ankara, Turkey
e-mail: cihan.yurdaydin@medicine.ankara.edu.tr

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_45

management of active CMV infection and for the assessment of latent HDV infection.

Keywords
Immunohistochemistry • Hepatitis A • Hepatitis B • Hepatitis C • Hepatitis D • Hepatitis E • Cytomegalovirus infection

List of Abbreviations

CHB	Chronic hepatitis B
CMV	Cytomegalovirus
HAV	Hepatitis A virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B early antigen
HBsAg	Hepatitis B surface antigen
HBV DNA	Hepatitis b virus DNA
HDAg	Hepatitis D antigen
HDV RNA	Hepatitis D virus RNA
IHC	Immunohistochemistry

Key Facts

1. Immunohistochemistry of viral hepatitis is based on antibody production in immunized animals or recombinant antibody production.
2. The developed antibodies target the virus or viral antigen, and these antigen-antibody systems form the base of several methods which include immunohistochemistry (IHC) but is not confined to IHC.
3. These methods may be useful for diagnosing and managing viral hepatitis of different causes, but they can also be used for research purposes.
4. Since liver biopsy is an invasive procedure and other means of liver disease assessment have developed in recent years, IHC is less performed for diagnosis and management although there may be exceptions to this.
5. On a research aspect, studies with antibodies against different viral proteins have contributed to our understanding of the biology and function of these viruses.

Definition of Words and Terms

ELISA (enzyme-linked immunosorbent assay)	A test that uses antibodies and color change to identify a substance. It is an analytic biochemistry assay that uses a solid-phase enzyme immunoassay (EIA) to detect the presence of a substance, usually an antigen, in a liquid sample. It is widely used as a diagnostic tool in medicine but has applications beyond medicine as well.

Flow cytometry	A biophysical technology was employed in cell counting, cell sorting, and biomarker detection. In this technique, cells are suspended in a stream of fluid and passed through an electronic detection apparatus. Flow cytometry is routinely used in diagnosis of several disorders, but has also applications in basic research and clinical practice.
Immunocytochemistry	A laboratory technique that is used to anatomically visualize the localization of a specific protein or antigen in cells by the use of a specific primary antibody that binds to it.
Immunofluorescence	A technique which uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell.
Immunohistochemistry	The process of detecting antigens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues.
Immunoprecipitation	The technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. This process can be used to isolate a particular protein from a sample containing many thousands of different proteins.
Mass spectrometry	An analytical technique that ionizes chemical species and sorts the ions based on their mass to charge ratio. Mass spectrum measures the masses within a sample. These spectra are used to determine the elemental or isotopic signature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules, such as peptides and other chemical compounds.
Western blot	An analytical technique used to detect specific proteins in a sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins.

Introduction

Viral hepatitis is liver inflammation due to a viral infection. The most common causes of viral hepatitis are the hepatotrop viruses A, B, C, D, and E. Viral hepatitis may lead to acute or chronic liver disease. All five viruses mentioned above can lead to acute viral hepatitis. In rare instances (<1% of acute hepatitis cases), acute viral hepatitis may be associated with hepatic encephalopathy and lead to fulminant hepatic failure which carries an exceptional high mortality risk. Viral hepatitis B, C, and D can lead to chronic hepatitis, and as such they continue to be an important

health problem as they can lead to complications associated with chronic liver disease including decompensated liver disease, hepatocellular carcinoma, and death from liver disease. Viral hepatitis B and C can be treated now with very potent antiviral medications which are effective in more than 95% of patients, whereas in chronic hepatitis D which is always associated with hepatitis B, treatment is less satisfactory. Nevertheless, treatment for all forms exists, and their proper diagnosis is key for their successful management (EASL Clinical Practice Guidelines 2012; EASL Recommendations on Treatment of Hepatitis C 2015; Yurdaydin et al. 2011). Liver biopsy has for long been regarded as the gold standard for diagnosing and correctly staging of liver disease due to viral hepatitis.

Today however, liver biopsy is rarely used for diagnosing viral hepatitis. Other biomarkers, such as liver enzymes, serological markers, and sensitive viral load determinations, are used for this purpose. Liver biopsy may be needed in rare instances to differentiate between acute hepatitis and reactivation of chronic hepatitis. On the other hand, in patients with chronic hepatitis, staging of liver disease is important as it may provide essential information regarding the clinical care of a patient, such as treatment prioritization, hepatocellular carcinoma screening, etc. In hepatitis B, where the natural history is divided into five different phases, i.e., the immune-tolerant, immune-reactive HBeAg-positive, inactive HBsAg carrier, HBeAg-negative, and HBsAg-negative phases (EASL Clinical Practice Guidelines 2012), the depiction of the correct stage the patient is in is crucial for the right management of the patient. Treatment is required in only the HBeAg-positive and HBeAg-negative phases, and liver biopsy may be required to decide on management. Treatment prioritization is currently an issue in many parts of the world for the management of chronic hepatitis C since new treatment options with interferon-free all-oral antiviral medications are for the most part expensive, and treatment therefore is not affordable in some countries. In others, treatment is prioritized for patients in greater need for treatment such as patients with advanced liver disease, posttransplant hepatitis C, etc. Thus, assessment of liver fibrosis continues to be very important. Despite this, liver biopsy is much less performed in patients with chronic hepatitis C (Castera et al. 2007). The main reasons for this are the increasing familiarity of practicing physicians with noninvasive assessment of liver disease and the accompanying comfort for patients and physicians alike of not performing an invasive procedure and that methods for noninvasive assessment of fibrosis have now been well validated for chronic hepatitis C.

In the past, immunohistochemistry of liver biopsy specimens was an integral part for management decisions. Hepatitis B core antigen (HBcAg) and hepatitis D antigen (HDAg) in the hepatocyte cytoplasm were indicative of active infection with hepatitis B or hepatitis delta, respectively (Hadziyannis 2011; Rizzetto 2009). These approaches are replaced by the comfort of relying on sensitive measurements of HBV DNA or HDV RNA by PCR-based methodology.

This chapter deals with IHC methods for hepatitis and their applications. It should be kept in mind while IHC is today much less often used or not used at all in the daily clinical practice of managing viral hepatitis, antibodies developed through

immunogens may have other applications and with these serve important purposes in research. In this context, it needs to be mentioned that these products can be used for techniques such as immunofluorescence, Western blotting, and others. The reader is asked to seriously take this into consideration when reading through the text as these techniques have been crucial contributors to our understanding of the biology of these viruses.

IHC for Hepatitis A Virus (HAV)

IHC for HAV is not performed in routine clinical practice since liver biopsy is not part of a routine diagnostic work-up of a patient with acute hepatitis. IHC staining in HAV infected chimpanzees disclosed that HAV stained positive for around 2 weeks after peak ALT elevation (Mathiesen et al. 1977). It may be expected that a similar pattern would apply for the human situation but no human data exists.

The cellular receptor for HAV is a member of T cell membrane proteins (TIMs) which are a family of transmembrane proteins expressed by various immune cells. TIM-1 had been identified as the cellular receptor for HAV (8). TIM-1 is a protein that in humans is encoded by the *HAVCR1* gene (Feigelstock et al. 1998). The TIM gene family, cloned first in a mouse model of asthma (McIntire et al. 2001), contains three members in humans which play important roles in the host immune response to viral infections but also mediate some immune regulatory functions in autoimmune diseases (Angiari et al. 2014; Xiao et al. 2012), asthma (McIntire et al. 2001; Xiao et al. 2015), and apoptosis (Lee et al. 2010).

TIM-1 may also function as a receptor or at least a cofactor for Ebola virus entry into the cell (Kondratowicz et al. 2011) and as a cofactor for dengue virus entry (Meertens et al. 2012).

For all these multitude of functions of TIM-1, several commercial antibodies are available which are mainly used for research purposes. A monoclonal antibody produced by immunizing rabbits with a synthetic peptide corresponding to residues surrounding Val24 of human TIM-1 protein exists, and its application is confined to immunohistochemistry and Western blotting. Another monoclonal antibody developed by immunizing mice with partially purified hepatitis A virus is available and is applicable for immunohistochemistry and ELISA systems. A similarly developed monoclonal antibody using purified hepatitis A virus as the immunogen is available for IHC.

IHC for Hepatitis B Virus (HBV)

HBV infection may lead to chronic hepatitis. The proportion of patients developing chronic hepatitis depends on the time of acquisition of hepatitis B infection. Mother to child vertical transmission may lead to chronic infection in around 90% of affected offspring, and chronicity may develop in 20–30% of patients confronted with HBV between ages 1 and 5. In later childhood, chronicity develops at a rate

similar to what is observed in adults, namely, 5%. Liver biopsy is practically not performed in patients with acute hepatitis B, but it is still performed, albeit less often than in the past, in patients with chronic hepatitis B (CHB). Liver biopsy is mostly performed in cases where the physician is not sure about the stage of liver disease and especially in places where transient elastography for assessing liver fibrosis is not available. The insurance body of a country responsible for reimbursement of HBV treatment may insist on a liver biopsy to avoid unnecessary treatment. In many centers around the globe, histological assessment of liver biopsies of patients with HBV includes IHC staining for hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg).

HBcAg is a product of the precore/core gene of HBV. HBcAg is required for packaging of viral DNA into core particles, a critical process in the life cycle of the virus (Seeger and Mason 2000). Nuclear localization of HBcAg in hepatocytes has been associated with no or minimal liver injury (Fig. 1) (Chu and Liaw 1987, 1992a; Mondelli et al. 1986) and with active HBV replication (Chu et al. 1997; Serinoz et al. 2003). Predominant cytoplasmic localization of HBcAg is associated with liver injury (Chu and Liaw 1992a; Sansonno et al. 1988). Positive HBsAg IHC staining (Fig. 2) is expected in patients with serum HBsAg positivity. A negative HBsAg staining under this condition may be observed in patients with self-limiting acute hepatitis or may be due to low amounts of intracellular HBsAg (Callea 1997). The use of avidin-biotin-peroxidase complex enhances immunohistochemical peroxidase-antiperoxidase techniques and increases sensitivity for IHC. Active hepatitis B infection with liver injury is characterized with HBsAg IHC staining associated with mostly cytoplasmic HBcAg staining, whereas inactive disease is associated with HBsAg staining with nuclear or absent HBcAg staining as mentioned also above (Thompson et al. 2010). The number and intensity of HBsAg staining positively correlate with quantitative HBsAg serum titers. However, IHC staining pattern may change according to the stage of hepatitis B virus infection. While high serum HBsAg titers observed mainly in HBeAg-positive CHB are associated with a membranous staining pattern, in HBeAg-negative CHB membranous staining is negligible or absent (Thompson et al. 2010).

The HBsAg gene contains three in frame “start” (ATG) codons that divide the gene into three sections: pre-S1, pre-S2, and S, responsible for the production of the large, middle, and small (pre-S1 + pre-S2 + S, pre-S2 + S, or S) antigens, respectively. The pre-S1 is a crucial part of the HBsAg for its cellular attachment to the hepatocyte.

HBxAg is a HBV protein with important function. It activates transcription of several host and viral genes (Seeger and Mason 2000); HBx protein is recruited onto HBV cccDNA and is involved in HBV replication (Levrero et al. 2009) and appears to have an important mediatory role in HBV-induced hepatocarcinogenesis (Neuveut et al. 2010). Antibodies have been developed to all of these hepatitis B proteins, and

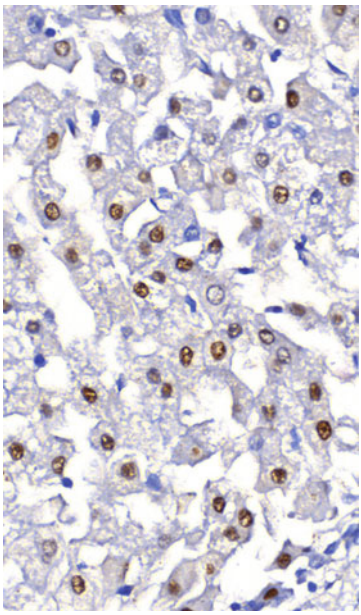

Fig. 1 Nuclear HBcAg staining in hepatocytes (streptavidin-biotin, ×400)

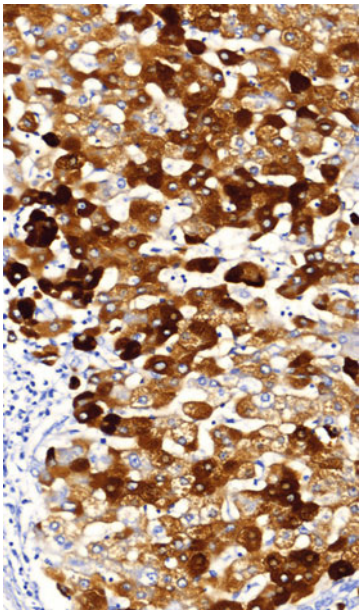

Fig. 2 Cytoplasmic HBsAg staining with IHC in hepatocytes (streptavidin-biotin, ×200)

Table 1 Antigen-antibody systems for assessing HBV proteins and their applications

Ag-Ab system	Product	Applications
HBsAg-Ab	Mouse monoclonal Ab	ELISA, FCM, IF, IP, IHC-P, WB
HBsAg-Ab	Rabbit polyclonal Ab	ELISA, IHC-F, IHC-P
HBsAg-Ab	Recombinant HBsAg protein	ELISA, WB
HBcAg-Ab	Mouse monoclonal Ab	ELISA, FCM, IF, IP, IHC-P, WB
HBcAg-Ab	Recombinant protein	ELISA, WB
HBeAg-Ab	Mouse monoclonal Ab	ELISA, WB,
HBeAg-Ab	Goat polyclonal Ab	ELISA, WB
HBV DNA Polymerase Ab	Rabbit polyclonal Ab	ELISA, ICC, IF, IHC-F, IHC-P, WB
HBV Polymerase Ab	Mouse monoclonal Ab	IF, IP, WB,
HB-pre-S1 Ab	Mouse monoclonal Ab	IF, IP, IHC-P, WB
HB-pre-S2 Ab	Mouse monoclonal Ab	ELISA, FCM, ICC/IF, IP, IHC-F, IHC-P
HBxAg-Ab	Mouse monoclonal Ab	ELISA, IP, IHC-F, IHC-P, WB

Abbreviations: *Ag* antigen, *Ab* antibody, *FCM* flow cytometry, *IF* immunofluorescence, *IP* immunoprecipitation, *IHC-F* immunohistochemistry of frozen samples, *IHC-P* immunohistochemistry of paraffin-embedded specimens, *ICC* immunocytochemistry, *WB* Western blot

they can be used in various applications. Table 1 provides antigen-antibody systems related to HBV antigens and their tested applications.

IHC for Hepatitis D Virus (HDV)

HDV-induced viral hepatitis represents the least encountered but most severe form of viral hepatitis. HDV can cause acute and chronic hepatitis. It needs the helper function of HBV to cause disease (Yurdaydin et al. 2011). HDV as a cause of liver disease was discovered by Mario Rizzetto and his colleagues through IHC detection of HDV in liver tissue of patients with chronic delta hepatitis (Rizzetto et al. 1977). HDV-IHC was for a long time used to define patients with active disease. Studies have revealed that HDV-HBV infection was mainly a disease caused by HDV. HDAg expression is mainly nuclear in location (Chu and Liaw 1992b) (Fig. 3). HBsAg staining is mainly cytoplasmic and almost always associated with HDAg expression, whereas HBcAg expression is less frequently observed than HDAg and HBsAg staining in patients with CDH (Kabaçam et al. 2011). HDAg semiquantitative staining frequency displayed correlation with ALT levels, whereas HBcAg and HBsAg staining did not show such a correlation confirming the crucial role of HDV in the mediation of liver injury associated with chronic delta hepatitis (Kabaçam et al. 2011). Despite the advances in diagnosing and assessing chronic delta hepatitis, HDV-IHC has been shown to be a sensitive marker of the existence of HDV without HBV in the posttransplant setting (Mederacke et al. 2012). This could be clinically

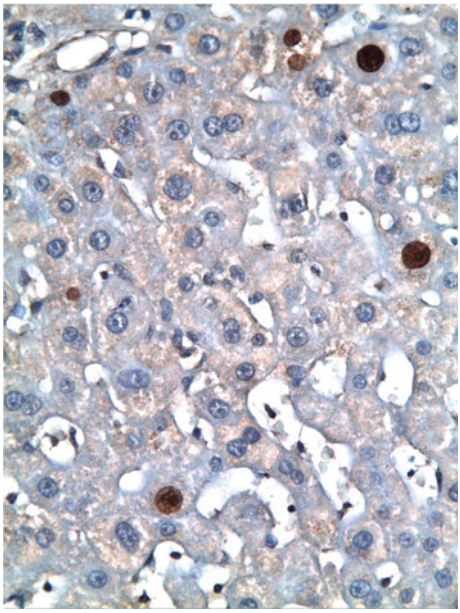

Fig. 3 Nuclear HDAg staining in hepatocytes (streptavidin-biotin, ×400)

important as it shows that HDV infection could be potentially "rescued" months after liver transplantation in case of HBV reinfection (Mederacke et al. 2012).

Delta-interacting protein A (DIPA), a cellular gene product, has been found to have homology to hepatitis delta virus antigen (HDAg). DIPA interacts with the viral antigen, HDAg, and can affect HDV replication in vitro. Rabbit polyclonal HDV antibodies have been developed through immunization of rabbits with conjugated synthetic peptide between 75 ∼ 105 amino acids from the center region of human DIPA. At least 29 hepatitis delta antigen-interacting protein A homolog antibodies from 17 antibody suppliers are available which can be used for western blood, immunofluorescence, and immunohistochemistry.

IHC for Hepatitis E Virus (HEV)

HEV, first documented as the causative agent of an outbreak of acute hepatitis cases in New Delhi, India, is a positive-sense single-stranded RNA virus which causes acute viral hepatitis in immunocompetent individuals (Kamar et al. 2014) but may lead to chronic hepatitis in immunocompromised patients (Kamar et al. 2008; Karma et al. 2011; Dalton et al. 2009). Its importance as a public health problem has been more and more recognized in recent years, thanks also to serological assays with improved sensitivity and specificity (Dalton et al. 2008). Acute hepatitis E during pregnancy is well known for increased morbidity and mortality of the mother and her offspring. HEV has a fecal-oral transmission route (Kamar et al. 2008). As liver biopsy is mainly not a diagnostic tool in HEV hepatitis, HEV IHC is not used for diagnostic purposes.

Table 2 Antigen-antibody systems for assessing HEV proteins and their applications

Ag-Ab system	Product	Applications
Anti-HEV Ag	Mouse monoclonal Ab	ELISA, IF, WB
Anti-HEV Ag	Rabbit polyclonal Ab	ELISA, WB
Anti-HEV ORF-3 Ag	Rabbit polyclonal Ab	IF, IHC-P, WB

Abbreviations: *Ag* antigen, *Ab* antibody, *IF* immunofluorescence, *IHC-P* immunohistochemistry of paraffin-embedded specimens, *WB* Western blot

Several polyclonal rabbit and monoclonal mouse antibodies to HEV exist. In Table 2, these are listed and their applications are provided.

IHC for Hepatitis C Virus (HCV)

HCV is associated with the highest rate of chronicity among hepatotrop viruses. The natural history of HCV is well described, and its management is currently undergoing a revolution which enables curative treatment in more than 90% with interferon- and mostly also ribavirin-free all-oral combination treatment regimens. Advances in HCV cell culture systems in the last decade have enabled an improved understanding of HCV virology, which has led to development of many new direct-acting antiviral drugs that target key components of virus replication (Webster et al. 2015).

Since these treatments are quite expensive in many parts of the world, prioritization for treatment of patients with more advanced liver disease is important. However, mostly noninvasive approaches mentioned in the introduction of this chapter are used to differentiate patients with advanced disease or cirrhosis from patients with mild disease. HCV-IHC is basically not used in the daily management of patients with chronic hepatitis C, but antibodies directed against different parts of the HCV virus have been and are still used for research purposes.

A detailed list of antibodies developed mainly for research purposes is provided in Table 3. Potential and tested applications of these antibodies are again provided.

IHC for Cytomegalovirus (CMV) Hepatitis

CMV infection-induced disease is more a problem of immunocompromised patients where it can be associated with significant morbidity and mortality although it may lead to disease reminiscent of infectious mononucleosis, also in immunocompetent individuals (Ghandi and Khanna 2004). Several methods are available for diagnosing CMV infection including serology, qualitative and quantitative polymerase chain reaction (PCR), pp65 antigenemia, culture, and histopathology. A commercial quantitative PCR assay which makes use of the recently developed WHO standard

Table 3 Antigen-antibody systems for assessing HCV proteins and their applications

Ag-Ab system	Product	Applications
Anti-HCV Ag-Ab	Mouse monoclonal Ab	ELISA, IHC-P,
Anti-HCV 1b core Ag	Rec HCV 1b core Ag protein	FS
Anti-HCV 1b core Ag	Mouse monoclonal Ab	ELISA, FCM, ICC/IF, IP, IHC-P, WB
Anti-HCV GT 1a core Ag	Rabbit polyclonal Ab to HCV core GT 1a	ELISA, WB
Anti-HCV GT 2a core Ag	Rec HCV 2a core Ag protein	FS
Anti-HCV GT 2b core Ag	Rec HCV 2b core Ag protein	ELISA, FS, WB
Anti-HCV GT 3a core Ag	Rec HCV 3a core Ag protein	FS
Anti-HCV GT 6A core Ag	Rec HCV 6a core Ag protein	ELISA, WB
Anti-HCV core NS3 + NS4	Polyclonal goat Ab	ELISA, IHC-F, WB
Anti-HCV GT 1 NS4	Rec HCV GT 1 NS4 protein	ELISA, WB
Anti-HCV GT 1a NS3	Rec HCV GT 1a NS3 protein	ELISA, FS, WB
Anti-HCV GT 1a NS5	Polyclonal goat Ab to HCV GT 1a NS5 protein	ELISA, FCM, IHC-P, WB
Anti-HCV GT 1b NS3	Rec HCV GT 1b NS3 protein	ELISA, WB
Anti-HCV GT 1b NS5	Rec HCV GT 1b NS5 protein	ELISA, WB
Anti-HCV GT 2 NS4	Rec HCV GT 2 NS4 protein	ELISA, WB
Anti-HCV GT 2a NS5	Rec HCV GT 2a NS5 protein	ELISA, FS, WB
Anti-HCV GT 2b NS3	Rec HCV GT 2b NS3 protein	ELISA, WB
Anti-HCV GT 2c NS3	Rec GT 2c NS3 protein	ELISA, WB
Anti-HCV GT 3 NS3	Rec HCV GT 3 NS3 protein	ELISA, FS, WB
Anti-HCV GT 3 NS4	Rec HCV GT 3 NS4 protein	ELISA, WB
Anti-HCV GT 5 NS4	Rec HCV GT 5 NS4 protein	ELISA, WB
Anti-HCV NS 5b	Rabbit polyclonal Ab	ELISA, IP, WB
Anti-HCV NS5b	Rec HCV NS5b protein	FS, MS

Abbreviations: *Ag* antigen, *Ab* antibody, *Rec* recombinant, *FCM* flow cytometry, *FS* functional studies, *IF* immunofluorescence, *IP* immunoprecipitation, *IHC-F* immunohistochemistry of frozen samples, *IHC-P* immunohistochemistry of paraffin-embedded specimens, *ICC* immunocytochemistry, *MS* mass spectroscopy, *WB* Western blot

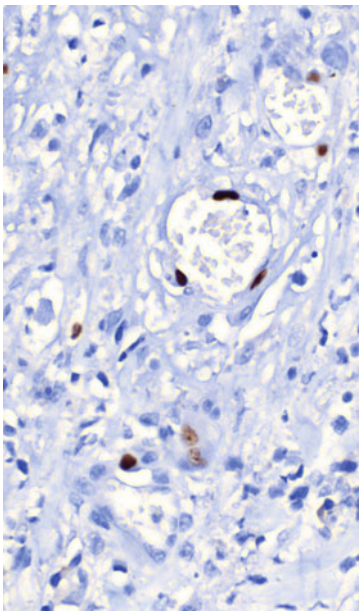

Fig. 4 Nuclear CMV inclusions showing strong positivity with anti-CMV (streptavidin-biotin, ×400)

is the preferred method for diagnosing active CMV infection. Liver biopsy with detection of CMV inclusion bodies or positive IHC for CMV appears to be the gold standard in tissue-invasive CMV infection (Kotton et al. 2013). Immunohistochemical detection of CMV in biopsy specimens has been reported to be more sensitive than demonstration of inclusion bodies or in situ hybridization detection of CMV (Lu et al. 2009; Colina et al. 1995). CMV can be detected both in the cytoplasm and nucleus of hepatocytes. In Fig. 4, a representative IHC staining of CMV Ag is shown, associated with nuclear CMV inclusions. Rabbit polyclonal and mouse monoclonal CMV antibodies from various companies are available for IHC-P, IHC-F, ELISA, Western blot, immunofluorescence, and functional assays (Table 4).

In conclusion, antibodies developed for IHC and other applications have been used more to better elucidate the biology of the various viruses mentioned in this chapter. The use of IHC methods for diagnostic purposes and for the management of patients is not used at all or is used less often than one or two decades ago. The increasing use of noninvasive markers for assessing disease severity is one important factor for this. Another factor deals with advances of other diagnostic tools. In this context, improvement in viral load determinations both with regard to standardization as well as in sensitivity and reproducibility and the availability and increased use of reliable commercial assays needs to be mentioned here. Despite these advances, IHC staining may still have a role for the diagnosis and management of HDV and CMV infections. This holds true especially in the posttransplant setting, where IHC is a useful adjunct for the diagnosis and management of active CMV infection and for assessing latent HDV infection.

Table 4 Antigen-antibody systems for assessing CMV proteins and their applications

Ag-Ab system	Product	Applications
Anti-CVM Ag	Mouse monoclonal Ab	ELISA, IF, FA, IHC-F, IHC-P, WB
Anti-CMV Ag	Rabbit polyclonal Ab	ELISA, WB
Anti-CMV Ag	Goat polyclonal Ab	ELISA, IHC, WB

Abbreviations: *Ag* antigen, *Ab* antibody, *IF* immunofluorescence, *FA* functional assay, *IHC-F* immunohistochemistry of frozen samples, *IHC-P* immunohistochemistry of paraffin-embedded specimens, *WB* Western blot

Summary Points

1. Immunohistochemistry (IHC) of liver specimens for the diagnosis and management of viral hepatitis is performed today less than in the past.
2. IHC of liver specimens is very rarely performed in acute viral hepatitis cases since most cases of acute viral hepatitis have a good prognosis and liver biopsy for diagnosis and management is not needed.
3. In patients with chronic viral hepatitis, assessment of severity of liver disease is requested both by physicians and patients. In most cases, especially in chronic hepatitis C cases, noninvasive tools are used for assessing liver disease severity, and liver biopsy is rarely performed in patients with chronic hepatitis C.
4. In chronic hepatitis B, liver biopsy to assess the stage of liver disease is more often used; still also in this condition, it is less often performed than in the past. During liver biopsy assessment, IHC for HBsAg and HBcAg is still performed in some centers, although their value in clinical practice is very limited.
5. IHC may still have a role in the post-liver transplant setting for the diagnosis and management of CMV hepatitis and for assessing latent HDV infection in the transplanted liver.
6. Antibodies developed for immunohistochemistry (IHC) have applications far beyond IHC and are applicable to various methods.

References

Angiari S, Donnarumma T, Rossi B, et al. TIM-1 glycoprotein binds the adhesion receptor P-selectin and mediates T cell trafficking during inflammation and autoimmunity. Immunity. 2014;40:542–53.

Callea F. Immunohistochemical techniques for the demonstration of viral antigens in liver tissue. Ric Clin Lab. 1997;18:223–31.

Castera L, Denis J, Babany G, Roudot-Thoraval F. Evolving practices of non-invasive markers of liver fibrosis in patients with chronic hepatitis C in France: time for new guidelines? J Hepatol. 2007;46:528–9.

Chu CM, Liaw YF. Intrahepatic distribution of hepatitis B surface and core antigens in chronic hepatitis B virus infection. Hepatocyte with cytoplasmic/membranous hepatitis B core antigen as a possible target for immune hepatocytolysis. Gastroenterology. 1987;92:220–5.

Chu CM, Liaw YF. Immunohistological study of intrahepatic expression of hepatitis B core and E antigens in chronic type B hepatitis. J Clin Pathol. 1992a;45:791–5.
Chu CM, Liaw YF. Intrahepatic expression of hepatitis B core and surface antigens in chronic hepatitis delta-virus infection. J Hepatol. 1992b;16:153–8.
Chu CM, Yeh CT, Chien RN, et al. The degrees of hepatocyte nuclear but not cytoplasmic expression of hepatitis B core antigen reflects the level of viral replication in chronic hepatitis B virus infection. J Clin Microbiol. 1997;35:102–5.
Colina F, Juca NT, Moreno E, et al. Histological diagnosis of cytomegalovirus hepatitis in liver allografts. J Clin Pathol. 1995;48:351–7.
Dalton HR, Bendall R, Ijaz S, Banks M. Hepatitis E: an emerging infection in developed countries. Lancet Infect Dis. 2008;8:698–709.
Dalton HR, Bendall R, Keane F, Tedder R, Ijaz S. Persistent carriage of hepatitis E virus in patients with HIV infection. N Engl J Med. 2009;361:1025–7.
EASL Clinical Practice Guidelines. Management of chronic hepatitis B virus infection. J Hepatol. 2012;57:167–85.
EASL Recommendations on Treatment of Hepatitis C. 2015;63:199–236.
Feigelstock D, Thompson P, Mattoo P, et al. The human homolog of HAVcr-1 codes for a hepatitis A virus cellular receptor. J Virol. 1998;72:6621–8.
Ghandi MK, Khanna R. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. Lancet Infect Dis. 2004;4:725–38.
Hadziyannis S. Milestones and perspectives in viral hepatitis B. Liver Int. 2011;31 Suppl 1:129–34.
Kabaçam G, Wedemeyer H, Savas B, et al. Role of immunohistochemistry for hepatitis D and hepatitis B virus in hepatitis delta. Liver Int. 2011;34:1207–15.
Kamar N, Selves J, Mansuy JM, et al. Hepatitis E virus and chronic hepatitis in organ-transplant recipients. N Engl J Med. 2008;358:811–7.
Kamar N, Dalton HR, Abravanel F, Izopet J. Hepatitis E virus infection. Clin Microbiol Rev. 2014;27:116–38.
Karma N, Garrouste C, Haagsma EB, et al. Factors associated with chronic hepatitis in patients with hepatitis E virus infection who have received solid organ transplants. Gastroenterology. 2011;140:1481–9.
Kondratowicz AS, Lennemann NJ, Sinn PL, et al. T-cell immunoglobulin and mucin domain 1 (TIM-1) is a receptor for Zaire Ebolavirus and Lake Victoria Marburgvirus. Proc Natl Acad Sci U S A. 2011;108:8426–31.
Kotton CN, Kumar D, Caliendo AM, et al. Updated international consensus guidelines on the management of cytomegalovirus in solid-organ transplantation. Transplantation. 2013;96:333.
Lee HH, Meyer EE, Goya S, et al. Apoptotic cells activate NKT cells through T cell Ig-like mucin-like–1 resulting in airway hyperreactivity. J Immunol. 2010;185:5225–35.
Levrero M, Pollicino T, Peterson J, et al. Control of cccDNA function in hepatitis B virus infection. J Hepatol. 2009;51:581–92.
Lu DYL, Qian J, Easley K, et al. Automated in situ hybridization and immunohistochemistry for cytomegalovirus detection in paraffin-embedded tissue sections. Appl Immunohistochem Mol Morphol. 2009;17:158–64.
Mathiesen LR, Feinstone SM, Purcell RH, et al. Detection of hepatitis A antigen by immunofluorescence. Infect Immun. 1977;18:524.
McIntire JJ, Umetsu SE, Akbari O, et al. Identification of *Tapr* (an airway hyperreactivity regulatory locus) and the linked *Tim* gene family. Nat Immunol. 2001;2:1109–16.
Mederacke I, Filmann N, Yurdaydin C, et al. Rapid early HDV RNA decline in the peripheral blood but prolonged intrahepatic hepatitis delta antigen persistence after liver transplantation. J Hepatol. 2012;56:115–22.
Meertens L, Carnec X, Lecoin MP, et al. The TIM and TAM families of phosphatidylserine receptors mediate dengue virus entry. Cell Host Microbe. 2012;12:544–57.
Mondelli M, Tedder RS, Ferns B, et al. Differential distribution of hepatitis B core and E antigens in hepatocytes: analysis by monoclonal antibodies. Hepatology. 1986;2:199–204.

Neuveut C, Wei Y, Buendia MA. Mechanisms of HBV-related hepatocarcinogenesis. J Hepatol. 2010;52:594–604.

Rizzetto M. HDV: 30 years after. J Hepatol. 2009;50:1043–50.

Rizzetto M, Canese MG, Arico S, et al. Immunofluorescence detection of new antigen-antibody system (delta/anti-delta) associated to hepatitis B virus in liver and in serum of HBsAg carriers. Gut. 1977;18:997–1003.

Sansonno DE, Fiore G, Bufano G, et al. Cytoplasmic localization of hepatitis B core antigen in hepatitis B virus infected livers. J Immunol Methods. 1988;109:245–52.

Seeger C, Mason WS. Hepatitis B virus biology. Microbiol Mol Biol Rev. 2000;64:51–68.

Serinoz E, Varli M, Erden E, et al. Nuclear localization of hepatitis B core antigen and its relations to liver injury, hepatocyte proliferation, and viral load. J Clin Gastroenterol. 2003;36:269–72.

Thompson AJV, Nguyen T, Iser D, et al. Serum hepatitis B surface antigen and hepatitis B e antigen titers: disease phase influences correlation with viral load and intrahepatic hepatitis B virus markers. Hepatology. 2010;51:1933–44.

Webster DP, Klenerman P, Dusheiko GM. Hepatitis C. Lancet. 2015;385:1124.

Xiao S, Brooks CR, Zhu C, et al. Defect in regulatory B-cell function and development of systemic autoimmunity in T-cell Ig mucin 1 (Tim-1) mucin domain-mutant mice. Proc Natl Acad Sci U S A. 2012;109:12105–10.

Xiao S, Brooks CR, Sobel RA, et al. Tim-1 is essential for induction and maintenance of IL-10 in regulatory B cells and their regulation of tissue inflammation. J Immunol. 2015;194:1602–8.

Yurdaydin C, Idilman R, Bozkaya H, Bozdayi AM. Natural history and treatment of chronic delta hepatitis. J Viral Hepat. 2011;17:749–56.

Phase Angle Bioelectrical Impedance Analysis (BIA) as a Biomarker Tool for Liver Disease

36

Cláudio Augusto Marroni, Daniella Miranda, Laura Boemeke, and Sabrina Alves Fernandes

Contents

Abstract

Cirrhotic patients may present body asymmetry and presence of ascites and edema, and the assessment of nutritional status is limited and fails when using the methods of anthropometric assessment. In addition, scores currently used, Model for End-Stage Liver Disease (MELD) and Child–Turcotte–Pugh, have limitations as to predict the prognosis of these patients. In this sense, as a complementary evaluation for liver disease, the analysis of the phase angle (PA) by bioelectrical impedance analysis (BIA) could be a new biological method to be used.

The BIA is a noninvasive method for evaluation of body composition, easy to perform, and fast, reproducible, and economical and can be performed in

C.A. Marroni (✉) • D. Miranda (✉) • L. Boemeke (✉)
Post Graduate Program in Medicine: Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil
e-mail: nmarroni@terra.com.br; dani_miranda28@hotmail.com; laurabboemeke@gmail.com

S.A. Fernandes (✉)
Post Graduate Program in Bioscience and Rehabilitation; Post Graduate Program in Rehabilitation and Inclusion, IPA Methodist University, Porto Alegre, RS, Brazil
e-mail: sabrinaafernandes@gmail.com

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_43

outpatient or inpatient. It tells us the nutritional status of patients by estimating the amount of lean body mass, fat mass, body water, and cell mass. The method also allows the assessment of patient's prognosis through the PA, which has been applied in patients with various diseases, including chronic liver disease. The phase angle varies according to the population and, in our environment, if we adopt the value of 5.4° as the cutoff point; values above represent a good prognosis and below this a poor prognosis.

Keywords

Liver diseases • Liver cirrhosis • Prognosis • Disease progression • Nutritional assessment

List of Abbreviations

AC	Arm circumference
AMC	Arm muscle circumference
APMT	Abductor pollicis muscle
BCM	Body cell mass
BIA	Bioelectrical impedance analysis
CCl4	Carbon tetrachloride
CHF	Congestive heart failure
CMR	Cardiac magnetic resonance
COPD	Chronic obstructive pulmonary disease
DEN	Diethylnitrosamine
EAT	Epicardial adipose tissue
ECW	Extracellular water
EF	Extracellular fluid
ES	Extracellular solids
FFM	Fat-free mass
FM	Fat mass
HAART	Highly active antiretroviral therapy
HCV	Hepatitis C virus
HGS	Hand grip strength
HIV	Human immunodeficiency virus
ICW	Intracellular water
MELD	Model for End-Stage Liver Disease
MF-BIA	Multiple-frequency bioimpedance analysis
PA	Phase angle
PCM	Protein caloric malnutrition
R	Resistance
SF-BIA	Single-frequency bioimpedance analysis
SPA	Standardized phase angle
TBARS	Thiobarbituric acid reactive substances
TBW	Total body water
Xc	Reactance
Z	Impedance

Key Facts of Child

- The Child score was originally proposed to evaluate the risk of cirrhotic patient subjected to portacaval anastomosis or esophageal transection.
- This prognostic model was developed by Child and Turcotte in 1964, but in the 1970s Pugh made a change in the score, replacing the variable "nutritional status" with "prothrombin time," thus creating the score we know today by Child or Child–Turcotte–Pugh score.
- The Child score is currently used to determine the prognosis, the response to treatment, and the need for liver transplantation.
- The Child–Pugh score includes three continuous variables (prothrombin time, bilirubin, and albumin) and two quantitative (ascites and hepatic encephalopathy). They are awarded points 1–3 according to the classification of each of the variables. The score is the sum of these points, ranging from 5 to 15, and the higher the score, the worse the prognosis.
  In general, patients with a score of 5 and 6 are classified as A, between 7 and 9 as B, and 10–15 as C.
- Regarding the MELD score, the Child score has the disadvantage of relying on subjective clinical severity variables (ascites and encephalopathy), which can hamper the classification of the patient.

Definitions of Words and Terms

Bioimpedance electrical	It is the device which indirectly measures the body composition of individuals by passing an electric current, which measures fat-free mass, fat mass, body cell mass, total body water, intracellular water, and extracellular water. In addition, the bioelectrical impedance provides the phase angle.
Phase angle	It evaluates the integrity, functionality, and cell membrane permeability. It is calculated by the resistance and reactance and provides information about the patient's prognosis.
Resistance	It is the opposition offered by the body to the passage of electric current and inversely related to water and electrolytes contained in body tissues.
Reactance	It is the capacitance (viability) of the properties of the cell membrane and may vary as a result of their integrity, function, and composition.
Multi-body model compartmentalization	It is an indirect method of assessing body composition, based on the quantification of fat mass, fat-free mass, minerals, water, tissue, blood, cell, and bone mass. Obtaining these data will depend on the model to be used.

Body asymmetry	It is the absence of proportionality of the body parts in a sagittal plane, such as the human body. It can be caused by fluid retention (ascites, edema) often present in patients with liver disease.
Prognostic marker	It is an indicator of the evolution of the patient's disease. It may be composed of complications from the disease.
Child–Turcotte–Pugh	It is a staging system and clinical classification of cirrhotic patients. It is considered the value of serum bilirubin, serum albumin, and prothrombin time; the presence of ascites; and the development of hepatic encephalopathy.
MELD score	It is a system currently used to allocate patients enlisted for liver transplant, which uses three laboratory parameters, and they are serum bilirubin, creatinine, and international normalized ratio (INR).
Protein calorie malnutrition	It is the consequence of insufficient intake of energy and protein. In liver disease patients, their presence is related to complications of the disease (fluid retention, hepatic encephalopathy) and symptoms (dysgeusia, lack of appetite, early satiety, abnormal bowel movements, nausea and/or vomiting).

Introduction

Patients with liver disease have a poor prognosis by the natural course of the disease and clinical complications arising from it along time (Parise et al. 2010).

Current methods for assessment and clinical staging of chronic liver disease are complex, objective, and/or subjective, such as the MELD score (*the Model for End-Stage Liver Disease)* and Child–Turcotte–Pugh, with reasonable reliability (Durand and Valla 2005; Huo et al. 2005). However, these methods do not contemplate or quantify important variables such as ascites, jaundice, encephalopathy, and disease severity (Botta et al. 2003). At the same time, patients should be evaluated sequentially, at different times, with clinical and/or laboratory tests, in order to establish the dynamic staging and prognosis. Thus, these methods are not fast, accurate, or instantaneous procedures, to respond faithfully to the actual state of the patient.

Chronic liver disease patients show numerous pathophysiological changes that compromise various organs and systems, as well as metabolic disorders, dysgeusia, severe depletion of skeletal muscles, and changes in hydration status (Fernandes et al. 2012). A constant feature, in variable order, is the nutritional deficits of macro-

and micronutrients, varying levels during the evolution of time, and disease staging, considering that these patients are malnourished per se, by the catabolic disease condition, and also have to follow a restrictive diet which further compromises the malnutrition framework (Müller et al. 1999; Matos et al. 2002).

Nutritional assessment of chronic liver disease by objective (anthropometry, body composition assessment, biochemical parameters, and evaluation of food consumption) and subjective (physical examination and the overall subjective assessment) methods is all partial, incomplete, discrepant, and not comparable, so it ends up being classified as unsuitable for the chronic liver disease patient (Fernandes et al. 2012; Ritter and Gazzola 2006; Gottschall et al. 2004; Donaghy 2002). There is not a standardized method considered gold standard for nutritional evaluation of chronic liver disease until now (Fernandes et al. 2012). Several nutritional assessment methods are often used together so that we can get an idea of the nutritional status of the patient resulting in clinical management and allowing directly intervention and improved nutritional status.

Among the methods used to assess the individual's nutritional status, the assessment by bioelectrical impedance analysis (BIA) was shown to be an accurate method to determine the components of body composition and proportions (fat mass, lean mass, body water, basal metabolic rate) and to establish the nutritional status of patients that exhibit no change in body symmetry. This compartmentalized evaluation of the body through the BIA (fat mass and fat-free mass) can provide, quantitatively, the actual nutritional status of the patient assessed.

On the other hand, chronic liver disease patients may show a change in body composition that affects the evaluation by BIA (Fig. 1), because as we will see later in more details, BIA assumes that the human body is a cylinder and that the cross-sectional areas represent the tissues of the organs analyzed by the passage of an electric current. Therefore, if the patient is overhydrated, the body lean mass is overestimated by modifying the result of the body evaluation (Kyle et al. 2004; Bera 2014). This is the greatest limitation of this method in chronic liver disease.

In the 1980s, it was found that BIA could assess the cellularity of living beings, through an observation of parameters obtained during the examination, placed in a special formula, and this does not depend on the body asymmetry observed in cirrhotic patients with edema and ascites. The result of this observation, calculated numerically, was called phase angle (PA) (Baumgartner et al. 1988).

Bioelectrical Impedance Analysis (BIA)

Bioelectrical impedance analysis is a noninvasive, inexpensive, and portable method that has been used mainly for body composition analysis (Barbosa-Silva et al. 2005). BIA is a safe technique and hazard-free that measures fat-free mass, fat mass, body cell mass, total body water, intracellular water, and extracellular water with an excellent consistency for repeated measurements (Bera 2014).

Different methods seek to evaluate body composition (Fig. 2). The classic model of two components of the body composition (2-C) divides the body into two parts,

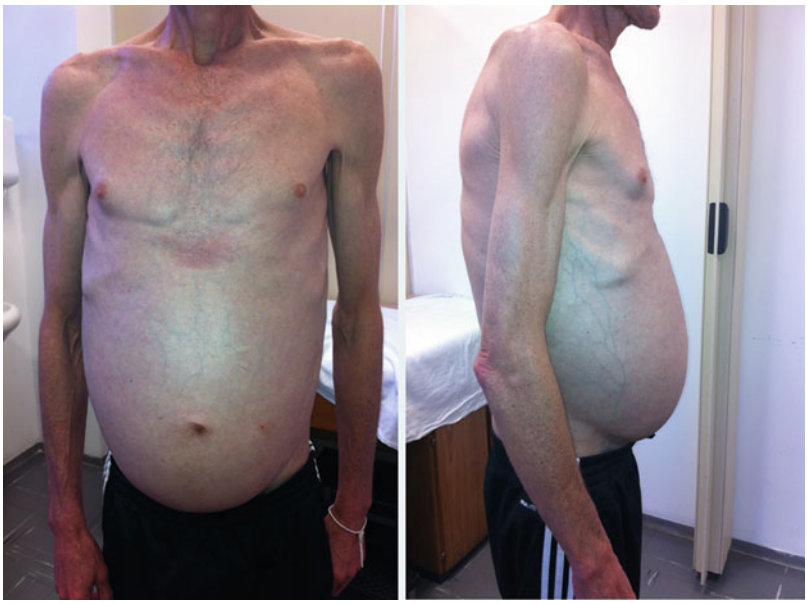

Fig. 1 Photo of a cirrhotic patient malnourished with body asymmetry. Body asymmetry in cirrhotic patient evidenced by the presence of ascites due to decompensated liver disease. Besides, it is possible to see the muscle depletion, common characteristic of the cirrhotic patients (Source: Author)

one of which consists of body fat and all the remaining tissues are collected and termed as fat-free mass (FFM).

Another model, the three components of the body composition (3-C), not only identifies the fat-free mass but also divides into two parts, liquid content (water) and remaining solids (predominantly proteins and minerals) (Cezar 2000).

More detailed than the previous models, the 4-component model, the cellular model, subdivides the fat-free mass into three basic or physiological compartments: body cell mass (BCM), water or extracellular fluid (ECF), and extracellular solids (ECS). So the fat-free mass (FFM) is defined as BCM + ECF + ECS and total fat mass as FFM – body weight (mass) (Cezar 2000).

Through the BIA method, it is possible to assess the body composition of all the proposed models. The BIA is an evaluation through which there is a passage of painless electric current through the organism, with low amplitude and low and high frequencies, applied by means of cables that are connected to electrodes or to conductive surfaces, which are placed in contact with the skin (Lukaski et al. 1985). The patient remains in dorsal decubitus position, with legs and hands placed parallel on the body. One electrode is placed on the dorsal hand, at the middle-finger level, and one in the wrist joint, both on the right side. Another pair of electrodes is placed on the dorsal foot, at the middle-toe level, and in the ankle joint, also on the right side (Fig. 3).

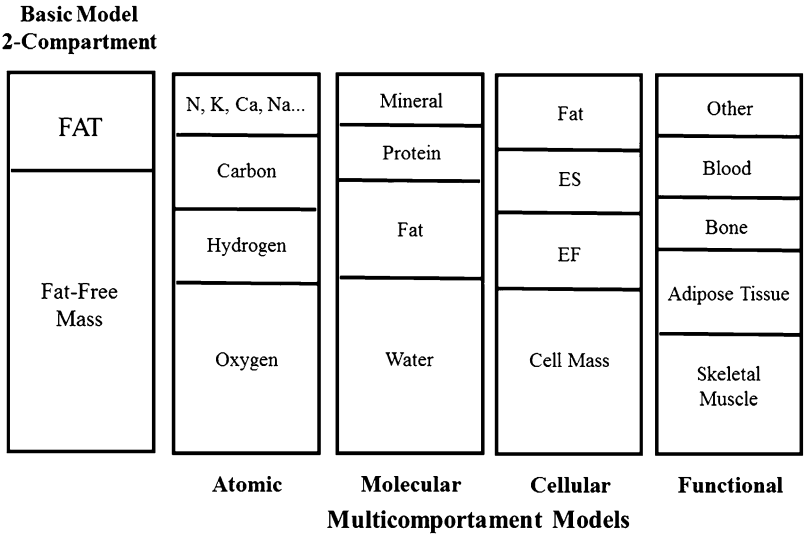

Fig. 2 Multi-body model compartmentalization. The figure above describes the basic two-compartment model that evaluates the fat mass and lean mass body composition. Atomic compartment model evaluates the body by dividing it into four compartments: compartment of minerals, compartment of carbon, compartment of hydrogen, and compartment of oxygen. The molecular model, in turn, evaluates the body according to the mineral content, protein, lipid, and water. The mobile model evaluates the contents of fat, extracellular fluid, extracellular solids, and cell mass. The functional model measures, in turn, the content of skeletal muscle, blood, bones, fat, and others. *EF* extracellular fluid, *ES* extracellular solids (Adapted from Ellis 2000)

The electric current passes through the body at a differential rate depending on body composition (Fig. 4) (Dehghan and Merchant 2008). The compartment known as fat-free mass consists of all that is not body fat and involves the following components: bone mineral content (≈7%), extracellular water (≈29%), intracellular water (≈44%), and visceral protein. Total body water (TBW) is a compartment which can be divided into extracellular water (ECW) and intracellular water (ICW). In turn, body cell mass (BCM) is the protein-rich compartment that is affected in catabolic states, and the loss of BCM is associated with unsatisfactory clinical results. Finally, the fat mass (FM) consists of total body fat, and it is obtained by subtracting fat-free mass (FFM) from total body weight (Mialich et al. 2014).

Considering the different composition of the body's compartments, the flow of electric current occurs differently in the muscle tissue compared to the fat, bone, and skin, and by this principle, it becomes possible to evaluate the resistance (R) and reactance (Xc) from the passage of electrical current. The muscle tissue contains a large amount of water and electrolyte and exhibits high conductivity and low electrical current strength. On the other hand, the fat, skin, and bones have low conductivity and high strength by containing small amount of fluid and electrolytes. Therefore, it identifies the resistance (R) and reactance (Xc), and it is possible to calculate the impedance (Z) and the phase angle (φ) (Lukaski et al. 1985). The phase

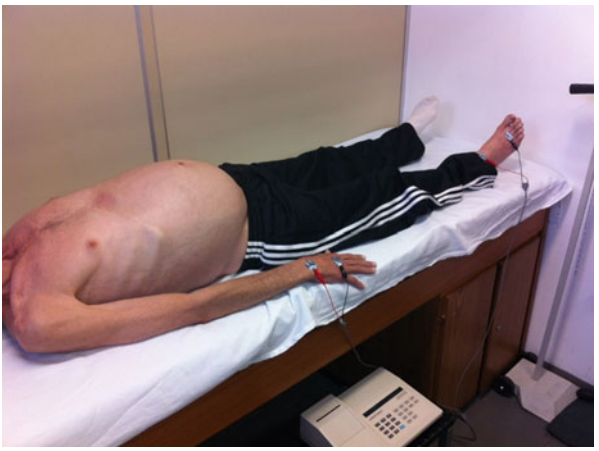

Fig. 3 Bioelectrical impedance analysis in cirrhotic patient. Cirrhotic patient being evaluated in outpatient care through bioelectrical impedance method. As we can see, the electrodes are placed on the hand and foot. The passage of an electric current through the organism can estimate the body composition and phase angle (Source: author, Fernandes S.)

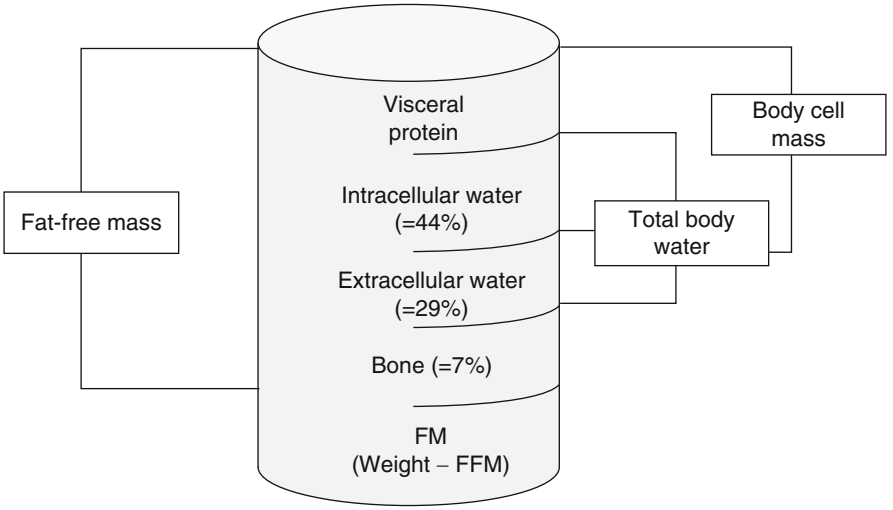

Fig. 4 Division of body compartments assessed by bioelectrical impedance. Among body compartments that can be evaluated using the bioelectrical impedance method is a fat-free mass, which is based on quantification of visceral protein, intracellular water, extracellular water, and bone mass, whereas fat mass corresponds to the subtraction of the fat-free mass of total body weight. The intracellular and extracellular water compartments provide the data of total body water and intracellular water, while the protein associated with visceral refers to the data of body cell mass (Source: Kyle et al. 2004)

angle between direct measurements that are provided by evaluating BIA shows itself as a prognostic indicator and mortality rates in several diseases, and its application in liver diseases is discussed later in this chapter.

Obtaining specific body compartment information depends on the type of electrical bioimpedance device used. Analysis of bioimpedance information obtained at

50 KHz electric current is known as single-frequency bioimpedance analysis (SF-BIA), which is the most used. In this case, the electrodes are placed on the hand and foot. SF-BIA allows estimating fat-free mass and total body water, but cannot determine differences in intracellular water (Kyle et al. 2004). Since it has a single frequency, this instrument may mask the interpretation of the data in tests in which the subject has altered body composition in some compartment. Thus, its use is not recommended in a situation of altered hydration (Mialich et al. 2014). Despite its limitations, the SF-BIA provides the phase angle (PA), which is related to the prognosis of cirrhotic patients (Fernandes et al. 2013).

Analysis of bioimpedance that is obtained at more than two frequencies is known as multiple-frequency bioimpedance analysis (MF-BIA) (Khalil et al. 2014). This method has more resources for assessment such as the determination of intracellular water since it involves currents with frequencies ranging from 5 to 100 kHz. Another resource of this instrument is its use as a marker of cell integrity, mentioned as a prognostic factor (Mialich et al. 2014).

Prior to completion of the bioelectrical impedance, the patient's preparation is important for the obtained result to be reliable. Among factors that can influence body composition stands out the consumption of drinks and food. Although food or fluid intake before BIA measurement affects total body water and extracellular water, a general agreement on the ideal amount of time between food and fluid intake and BIA measurements has yet to be consolidated. For this reason undertaking an overnight fasting is recommended as a routine standardization technique before impedance measurements (Dehghan and Merchant 2008).

The practice of exercise before taking a test may also influence body composition analysis. Although exercise of mild intensity may not affect BIA measurements, moderate and intensive exercise before measurements may change the measured impedance by different mechanisms (Garby et al. 1990). Therefore, in order to minimize the risk of error in the assessment by BIA, moderate to intense physical exercise between 2 and 3 h prior to testing is not recommended.

Furthermore, it is noted that the body asymmetry should be considered for evaluation of patients by bioelectrical impedance. It is understood by physical asymmetry in the absence of proportionality of the body parts in a sagittal plane, such as the human body. Thus, patients with ascites and edema, morbidly obese patients, pregnant women, and amputees will present change of body fluids. It should be noted that even if the patient is asymmetrical, there is no change in phase angle in these cases, which will be addressed ahead.

Phase Angle

The use of the BIA has shown efficiency in the measurement of body compartments in various clinical situations such as malnutrition, trauma, pre- and postoperative, compensated liver disease, renal dialysis, and cancer (Kyle et al. 2004).

Changes in body shape may influence the results of the examination. In these situations, the use of BIA may be more advisable to obtain better results. Recalling

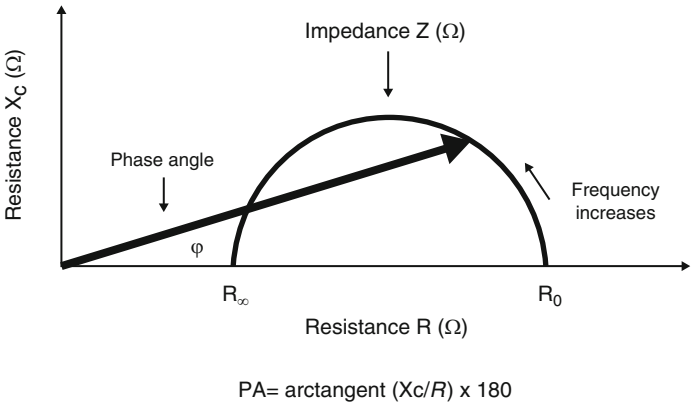

Fig. 5 Phase angle. **PA = arctangent (Xc/*R*) x 180.** Formula and geometric distribution of the formation of PA and its relationship with resistance, reactance, impedance, and frequency of the applied current (Source: Kyle et al. 2004)

that BIA is based on the body symmetry theory, where the level of hydration and percentage body fat are constant, when faced with different situations, such as age, ethnicity, body shape, or various clinical conditions, this method does not have "universal equations," used in all situations, requiring another reference point as a parameter (Barbosa-Silva and Barros 2005a; Barbosa-Silva et al. 2003).

Given these differences, the clinically established bioelectrical impedance parameter is the phase angle (PA). The phase angle is calculated by the formula that considers the resistance (R) and reactance (Xc) (Fig. 5).

The PA gained popularity in recent years, because it shows to be highly predictive of clinical outcome in a variety of diseases. Recent studies report that the PA values correlate well with clinical outcome parameters of the studied disease (Kyle et al. 2004). There are numerous diseases evaluated by PA, such as cancer, HIV, lung disease, heart disease, and others.

The phase angle is a fast method, is applicable in the clinic, and reflects the vitality and cell integrity, where higher values indicate cellular activity preserved (Máttar 1996; Norman et al. 2012). In healthy subjects, the PA can vary between 6° and 7° (Bosy-Wesrphal et al. 2006). Different cutoff values are used based on reference values for age and gender. Standardized PA (SPA) values exist for the Swiss, German, American, and Brazilian populations. The SPA can be used to compare results among different populations and to correlate an SPA with a particular disease, as reported by Barbosa-Silva et al. (2008), in the Brazilian population. The results of this study allow us to use the SPA values as parameters for other several diseases.

Phase Angle and Chronic Liver Disease

It is noted that the preserved function of the cells is closely linked to the nutritional status of cirrhotic individual, and it became an independent marker of mortality.

Selberg and Selberg (2002), in a prospective study of 305 patients with cirrhosis, correlated the PA with muscle mass, muscle strength, and survival rates. They observed that patients with a PA equal or lower than 5.4° showed lower survival rates than those with PA values above 6.6°. Variables such as total body potassium, anthropometric measurements, and BIA were evaluated separately; however, only the PA proved to be an isolated predictor of survival.

In a retrospective study, Pirlich et al. (2000) analyzed 41 cirrhotic patients (20 with ascites and 21 without) through BIA, considered as the reference method. The study shows that the PA is a tool able to detect body cellular mass and identify its decrease in cirrhotic patients. The PA offers reliable PCM estimates even when used in patients with large amount of ascites, proving to be superior to commonly used techniques, showing that the PA becomes an important prognostic marker.

A cohort that assessed 66 cirrhotic patients stratified by their clinical condition through the Child–Pugh score, followed up for a 17-month period, established PA for this population in 5.18°. Patients with values below this angle were considered to have poor prognosis and shorter survival rates. It is worth highlighting that as the patient's clinical situation worsened, the PA decreased, showing a prognostic value (Peres et al. 2012).

Corroborating with these findings, we assessed the nutritional status of 129 cirrhotic patients through different methods and demonstrated that the only method able to correlate malnutrition with the liver disease's staging, evaluated through the Child–Turcotte–Pugh classification, is PA. We set the PA cutoff point as 5.4°, same as Selberg and Selberg (2002), in which patients with values below this discriminatory level showed a worse prognosis. We should point out the discrepancies between the results of different evaluation methods (anthropometry, hand grip strength (HGS), and BIA), used to diagnose PCM, once the diagnosis for malnutrition may vary from 5.4% to 69.3% in the same population, depending on the assessment method employed (Fernandes et al. 2012). PA analysis demonstrated sensitivity and specificity when compared to BIA and HGS, with values of 68.9–70.0% and 49.2–56%, respectively (Fernandes et al. 2012).

Later, another study performed in our center evaluated 195 cirrhotic patients, reinforcing the idea that the PA is a good prognostic marker when compared to other methods, as it is the only one correlated with the real clinical condition of the patient (Fernandes et al. 2013).

Recently, Ruiz Margain et al. (2015) assessed 249 compensated cirrhotic patients in a prospective cohort study with a 48-month follow-up period. The PA cutoff point for malnutrition was lower or equal to 4.9°, the cutoff based in a pilot study. This study also concluded that PA is a good prognostic marker, associating PCM with mortality rate.

A cohort study conducted in our center evaluated 32 cirrhotic patients enlisted for liver transplant (Aydos et al. 2016). The patients were evaluated on the moment before the transplant and 1, 6, and 12 months after surgery. The assessment of nutritional status was performed applying diagnostic procedures in sequence: anthropometry, non-dominant hand grip strength, abductor pollicis muscle (APMT), and PA. Methods that better demonstrated the real prevalence of malnourished patients before transplantation were PA (25%), arm muscle circumference (AMC) (21.9%),

and arm circumference (AC) (18.8%). The percentage of malnourished patients was significantly higher after 1 month of transplantation when compared to the percentage in 6 months and 1 year after transplantation. Among these methods, the one that followed the disease's staging was PA, because as the patient improved, PA accompanied this increase. It was suggested that the PA could be widely used with this population since the results are consistent, reliable, and reproducible.

Wagner et al. (2011) evaluated nutritional methods that informed the nutritional status of 71 posttransplantation patients. Patients were divided into three groups according to the time since their transplantation: 5 years, between 5 and 10 years, and over 10 years. They used the PA cutoff point as below 5° in order to diagnose malnutrition. The PCM diagnosis was made in 81.2%, 31.6%, and 31.7% in each group, respectively ($p = 0.008$). In this study, PA showed a higher prevalence of malnutrition among the population of patients in the first years after liver transplantation.

Recently, Ruiz-Margáin et al. (2016) analyzed the clinical and nutritional status of 79 consecutive cirrhotic patients, prospectively, on intensive care unit. Sequentially, clinical, laboratory, and nutritional assessments (with BIA phase angle and measure of arm muscle circumference) were made to determine its evolution and prognosis. Evaluations were daily until discharge or death. All patients who died were malnourished. The PA decreased in patients with major complications, in the most severe ones, and in those who died. The decrease in PA in the first 24 h was associated with higher mortality. The PA has been shown as an early biomarker for prognosis that would make it useful as part of an initial full real-time assessment of these patients.

Analyzing patients with chronic hepatitis by the hepatitis C virus (HCV) with advanced fibrosis, PA/BIA was shown to be a predictor of the development of fibrosis, since for each degree of reduction of PA, there is a fourfold increase in the risk of fibrosis (de Souza et al. 2016). In patients with chronic C virus hepatitis in antiviral treatment, the reduction of PA/BIA joined to the increase of the adverse effects of this therapy (Kahraman et al. 2010).

The BIA is feasible in any living being and the PA calculation as well. Therefore, we use it in our research group, BIA and PA in rats with cirrhosis induced by carbon tetrachloride (CCl4), and observed that the values of PA decreased with the worsening of animals for disease progression (Fig. 6). Under that circumstance the cell membranes are compromised by lipoperoxidation and were confirmed by TBARS technique. In similar experiments using the model of cirrhosis diethylnitrosamine (DEN), we obtained the same results with reduced PA and its recovery, with improvement of animals by the use of antioxidants such as melatonin (Bona et al. 2012). In secondary biliary cirrhosis produced in rats by ligation of common bile duct, melatonin improved morphological and nutritional parameters, and the phase angle of the bioelectrical impedance has increased along with these improvements (Marroni et al. 2016).

The electrical conductivity in biological tissues is virtually ionic, meaning that the electrical charges were transferred to the ionization salts. Thus, the organic conductivity is directly proportional to the volume of body fluid.

Additionally, muscle strength and phase angle correlate, which is suggestive of a lower phase angle being associated with decreasing functioning status.

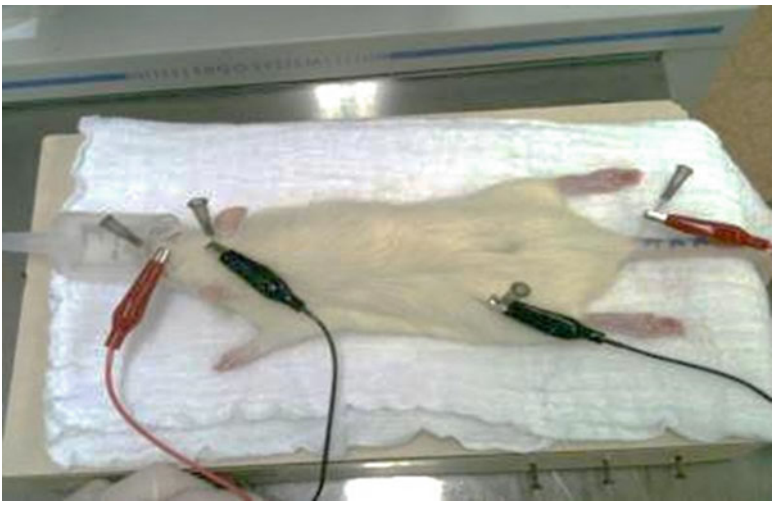

Fig. 6 Phase angle evaluation in animals. Using the monofrequential BIA in cirrhotic mouse induced by carbon tetrachloride (CCl4). Animals are anesthetized for the technique to be performed (Source: author, Fernandes S.)

Several markers have been associated with sarcopenia in the elderly, including bioelectrical indexes. Phase angle (PA) is an impedance parameter and has been suggested as an indicator of cellular death. Thus, the relationship between PA, muscle mass, and strength was investigated in 207 elderly participants (mean age 76.2 ± 6.7years) admitted for multidimensional geriatric evaluation. Muscle strength by grip strength using a handheld dynamometer and muscle mass were measured by bioimpedentiometer. PA was calculated directly with its arctangent (resistance/reactance × 180°/π). Linear relationship among muscular mass and strength and with clinical and biochemical parameters, including PA at uni- and multivariate analyses, was performed. Linear regression analysis demonstrated that lower level of PA is associated with reduction in grip strength ($y = 3.16 + 0.08x$; $r = 0.49$; $p < 0.001$) and even more with muscle mass ($y = 3.04 + 0.25x$; $r = 0.60$; $p < 0001$). Multivariate analysis confirms these relationships (grip strength $\beta = 0.245$, $p = 0.031$; muscular mass $\beta = 0.623$, $p < 0.01$). Thus, PA is inversely related to muscle mass and strength in elderly subjects, and it may be considered a good bioelectrical marker to identify elderly patients at risk of sarcopenia (Basile et al. 2014).

Phase angle is an objective bedside nutritional marker reflecting the integrity of cellular membranes and tissue homeostasis, translating into nutritional status and suitable for daily assessment in cirrhotic patients; thus, it could be a useful tool in the hospitalized population.

The PA has become an important prognostic marker in various clinical conditions in which integrity of cell membrane is compromised and there is a change in the balance of fluids (Kahraman et al. 2010). Studies suggest that PA is an important tool to evaluate the clinical outcome or disease progression. (Llames et al. 2013).

Potential Application of the Method for Other Diseases' Prognosis and Conditions

The phase angle has been studied for application as a prognostic indicator in several other clinical conditions, such as cancer, acquired human immunodeficiency virus (HIV), and chronic obstructive pulmonary disease (COPD); surgical patients; and kidney dialysis patients.

Gupta et al. (2004a) showed that PA is a powerful predictor of survival when compared to traditional parameters of nutritional assessment, such as albumin, prealbumin, and transferrin in patients with advanced pancreatic cancer. This study identified a cutoff for PA 5.0°. In a similar study conducted in patients with advanced lung cancer, there was a lower survival rate in patients with PA $\leq$ 4.5° (Toso et al. 2000). The same trend was observed in the use of PA as a predictor of mortality of patients with colorectal cancer, and patients presenting PA $>$ 5,57° had an average survival rate five times higher than those with PA below this cut point (40.4 months vs. 8.4 months) (Gupta et al. 2004b).

Regarding HIV-infected patients on highly active antiretroviral therapy (HAART), Schwenk et al. (2000) noted that PA has a strong ability to predict survival and clinical outcome, regardless of the level of immunodeficiency and viremia.

When evaluating a population composed of 225 presurgical individuals, Barbosa-Silva and Barros (2005b) found that weight loss greater than 10%, the subjective global assessment, nutritional risk assessment, extracellular mass/body cell mass, and PA were prognostic factors significantly associated with postoperative complications in the crude analysis. However, after adjusting for sex, age, marital status, tumor, and preoperative infections, only the PA remained as a prognostic factor.

Regarding heart disease, PA also appears to be a good predictor of survival (Doesch et al. 2010; G. Brenta et al. 2011) and a marker of severity of congestive heart failure (CHF) (Castillo et al. 2007). Doesch et al. (2010) investigated the association between PA and the epicardial adipose tissue (EAT) quantified by cardiac magnetic resonance (CMR) in 41 patients with CHF and 16 controls. CHF patients showed a decrease of PA (vs. 5.5° 6.4°, $P < 0.02$) when compared to controls. Linear regression analysis showed a significant correlation TAE index with PA, and the ROC curve showed good predictive performance PA and TAE regarding cardiac death (Doesch et al. 2010). Brenta et al. (2011), considering the plasma levels of triiodothyronine (T3) as a predictor of mortality in patients with CHF, studied its association with PA. In this study the authors found that the lowest tertile of T3 was associated with more advanced CHF and the lower PA values. Castillo et al. (2007), evaluating 243 patients with CHF, noted that PA was positively correlated with functional capacity, that is, the worse the functional capacity, the lower was the PA.

Conclusion

Although there are few studies about the PA as a prognostic factor in liver diseases, the articles published to date indicate that PA is an important predictor of mortality and disease progression in liver cirrhosis and hepatitis C virus.

The PA determined by the BIA in the evaluation of chronic liver disease patients shows itself as easy, inexpensive, reproducible, and reliably free of complications, which ultimately become an important element in determining the prognosis of the disease and can be used sequentially and repetitively to follow-up.

However, the PA obtained from the BIA is a value related to a given normal population which has not been universally characterized; therefore, studies should be carried out for new population cutoff determination of each population.

Summary Points

- Bioelectrical impedance analysis (BIA) is an important method of assessing body composition of healthy individuals.
- The BIA is a simple test, easy, economical, feasible in the hospital or clinic, and reproducible with reliability.
- In order to assess the body composition, the bioelectrical impedance to be held in rest conditions, without the individual, has practiced moderate or intense physical activity between 2 and 3 h previous to the test and without ingested food or liquids 12 h before the test.
- Phase angle (PA)/BIA is a biological marker of prognosis in patients with cirrhosis or hepatitis C virus.
- The PA of the bioelectrical impedance is a biological marker of prognosis and progression of various diseases.
- Population studies should be done to determine the value of the PA of BIA in these populations, which will serve as parameters for prognostic assessment of diseases.
- The PA, in our experience, has a cutoff value of 5.4° to chronic liver disease.

References

Aydos MED, Fernandes SA, Nunes FF, et al. One-year follow-up of the nutritional status of patients undergoing liver transplantation. Nutr Hosp. 2016;33:8–13.

Barbosa-Silva MC, Barros AJ. Bioelectrical impedance analysis in clinical practice: a new perspective on its use beyond body composition equations. Curr Opin Clin Nutr Metab Care. 2005a;8(3):311–7.

Barbosa-Silva MC, Barros AJ. Bioelectric impedance and individual characteristics as prognostic factors for post-operative complications. Clin Nutr. 2005b;24:830–8.

Barbosa-Silva MC, Barros AJ, Post CL, et al. Can bioelectrical impedance analysis identify malnutrition in preoperative nutrition assessment? Nutrition. 2003;19(5):422–6.

Barbosa-Silva MC, Barros AJ, Wang J, et al. Bioelectrical impedance analysis: population reference values for phase angle by age and sex. Am J Clin Nutr. 2005;82(1):49–52.

Barbosa-Silva MC, Barros AJ, Larsson E. Phase angle reference values for Brazilian population. Int J Body Compos Res 2008;6: 67–68.

Basile C, Della-Morte D, Cacciatore F, et al. Phase angle as bioelectrical marker to identify elderly patients at risk of sarcopenia. Exp Gerontol. 2014;58:43–6.

Baumgartner RN, Chumlea WC, Roche AF. Bioelectrical impedance phase angle and body composition. Am J Clin Nutr. 1988;48:16–23.

Bera TK. Bioelectrical impedance methods for noninvasive health monitoring: a review. J Med Eng. 2014;2014:381251.

Bona S, Moreira ACJ, Oliveira MS, et al. Caracterização do modelo experimental de carcinoma hepatocelular por Indução Química em Ratos. Rev HCPA. 2012;32(Suppl):151.

Bosy-Westphal A, Danielzik S, Dörhöfer RP, et al. Phase angle from bioelectrical impedance analysis: population reference values by age, sex, and body mass index. JPEN J Parenter Enteral Nutr. 2006;30(4):309–16.

Botta F, Giannini E, Romagnoli P, et al. MELD scoring system is useful for predicting prognosis in patients with liver cirrhosis and is correlated with residual liver function: a European study. Gut. 2003;52(1):134–9.

Brenta G, Thierer J, Sutton M, et al. Low plasma triiodothyronine levels in heart failure are associated with a reduced anabolic state and membrane damage. Eur J Endocrinol. 2011; 164(6):937–42.

Castillo LM, Colín ER, Orea AT, et al. Bioelectrical impedance and strength measurements in patients with heart failure: comparison with functional class. Nutrition. 2007;23(5):412–8.

Cezar C. Alguns aspectos básicos para uma proposta de taxionomia no estudo da composição corporal, com pressupostos em cineantropometria. Rev Bras Med Esporte. 2000;6(5):188–93.

de Souza DM, Santos LA, Gondo FF, et al. Phase angle is associated with advanced fibrosis in patients chronically infected with hepatitis C virus. Life Sci. 2016;154:30–33. pii: S0024–3205 (16)30112–6.

Dehghan M, Merchant AT. Is bioelectrical impedance accurate for use in large epidemiological studies? Nutr J. 2008;7:26.

Doesch C, Suselbeck T, Leweling H, et al. Bioimpedance analysis parameters and epicardial adipose tissue assessed by cardiac magnetic resonance imaging in patients with heart failure. Obesity (Silver Spring). 2010;18(12):2326–32.

Donaghy A. Advances in liver disease: alcoholic hepatitis, non-cirrhotic portal fibrosis and complications of cirrhosis. J Gastroenterol Hepatol. 2002;17:462–6.

Durand F, Valla D. Assessment of the prognosis of cirrhosis: Child–Pugh versus MELD. J Hepatol. 2005;42:100–7.

Ellis KJ. Human body composition: in vivo methods. Physiol Rev. 2000;80(2):649–80.

Fernandes SA, Bassani L, Nunes FF, et al. Nutritional assessment in patients with cirrhosis. Arq Gastroenterol. 2012;49(1):19–27.

Fernandes SA, Gonzalez MC, Bassani L, et al. Is the phase angle, a prognostic indicator for nutritional status in cirrhotic patients? J Antivir Antiretrovir. 2013;S3:004.

Garby L, Lammert O, Nielsen E. Negligible effects of previous moderate physical activity and changes in environmental temperature on whole body electrical impedance. Eur J Clin Nutr. 1990;44(7):545–6.

Gottschall CBA, Álvares-da-Silva MR, Camargo ACR, et al. Avaliação nutricional de pacientes com cirrose pelo vírus da hepatite C: a aplicação da calorimetria indireta. Arq Gastroenterol. 2004;41:220–4.

Gupta D, Lis CG, Dahlk SL, et al. Bioelectrical impedance phase angle as a prognostic indicator in advanced pancreatic cancer. Br J Nutr. 2004a;92(6):957–62.

Gupta D, Lammersfeld CA, Burrows JL, et al. Bioelectrical impedance phase angle in clinical practice: implications for prognosis in advanced colorectal cancer. Am J Clin Nutr. 2004b; 80(6):1634–8.

Huo TI, Wu JC, Lin HC, et al. Evaluation of the increase in model for end-stage liver disease (DeltaMELD) score over time as a prognostic predictor in patients with advanced cirrhosis: risk factor analysis and comparison with initial MELD and Child-Turcotte-Pugh score. J Hepatol. 2005;42:826–32.

Kahraman A, Hilsenbeck J, Nyga M, et al. Bioelectrical impedance analysis in clinical practice: implications for hepatitis C therapy BIA and hepatitis C. Virol J. 2010;7:191.

Khalil SF, Mohktar MS, Ibrahim F. The theory and fundamentals of bioimpedance analysis in clinical status monitoring and diagnosis of diseases. Sens (Basel). 2014;14(6):10895–928.

Kyle UG, Bosaeus I, De Lorenzo AD, et al. Composition of the ESPEN working group. Bioelectrical impedance analysis – part I: review of principles and methods. Clin Nutr. 2004; 23(5):1226–43.

Llames L, Baldomero V, Iglesias ML, et al. Values of the phase angle by bioelectrical impedance; nutritional status and prognostic value. Nutr Hosp. 2013;28(2):286–95.

Lukaski HC, Johnson PE, Bolonchuk WW, et al. Assessment of fat-free mass using bioelectrical impedance measurements of the human body. Am J Clin Nutr. 1985;41(4):810–7.

Marroni NP, Colares JR, Schemitt EG, et al. Melatonin mitigates the Nutritional and Morphological changes of rat tongue with secondary biliary cirrhosis induced by ligation of the common bile duct. In: The International Liver Congress, 2016, Barcelona. Journal of Hepathology. 2016; 64:S317–S318.

Matos C, Porayko MK, Francisco-Ziller N, et al. Nutrition and chronic liver disease. J Clin Gastroenterol. 2002;35:391–7.

Máttar JA. Application of total body bioimpedance to the critically ill patient. Brazilian Group for Bioimpedance Study. New Horiz. 1996;4(4):493–503.

Mialich MS, Sicchieri JMF, Jordao Jr AA. Analysis of body composition: a critical review of the use of bioelectrical impedance analysis. Int J Clin Nutr. 2014;2(1):1–10.

Müller MJ, Böttcher J, Selberg O, et al. Hypermetabolism in clinically stable patients with cirrhosis. Am J Clin Nutr. 1999;69:1194–201.

Norman K, Stobäus N, Pirlich M, et al. Bioelectrical phase angle and impedance vector analysis – clinical relevance and applicability of impedance parameters. Clin Nutr. 2012;31(6):854–61.

Parise ER, de Oliveira AC, de Carvalho L. Cirrose Hepática. In: Mattos AA, Dantas-Corrêa EB, editors. Tratado de hepatologia. 1st ed. Rio de Janeiro: Rubio; 2010. p. 429–37.

Peres WA, Lento DF, Baluz K, et al. Phase angle as a nutritional evaluation tool in all stages of chronic liver disease. Nutr Hosp. 2012;27(6):2072–8.

Pirlich M, Schütz T, Spachos T, et al. Bioelectrical impedance analysis is a useful bedside technique to assess malnutrition in cirrhotic patients with and without ascites. Hepatology. 2000;32:1208–15.

Ritter L, Gazzola J. Avaliação nutricional no paciente cirrótico: uma abordagem objetiva, subjetiva ou multicompartimental? Arq Gastroenterol. 2006;43:66–70.

Ruiz-Margáin A, Macías-Rodríguez RU, Duarte-Rojo A, et al. Malnutrition assessed through phase angle and its relation to prognosis in patients with compensated liver cirrhosis: a prospective cohort study. Dig Liver Dis. 2015;47:309–14.

Ruiz-Margáin A, Macías-Rodríguez RU, Chi-Cervera L, et al. Phase angle as an early nutritional marker of short-term outcome in hospitalized patients with cirrhosis. In: The International Liver Congress, 2016, Barcelona. Journal of Hepathology. 2016;64:S253.

Schwenk A, Beisenherz A, Römer K, et al. Phase angle from bioelectrical impedance analysis remains an independent predictive marker in HIV-infected patients in the era of highly active antiretroviral treatment. Am J Clin Nutr. 2000;72(2):496–501.

Selberg O, Selberg D. Norms and correlates of bioimpedance phase angle in healthy human subjects, hospitalized patients, and patients with liver cirrhosis. Eur J Appl Physiol. 2002; 86(6):509–16.

Toso S, Piccoli A, Gusella M, et al. Altered tissue electric properties in lung cancer patients as detected by bioelectric impedance vector analysis. Nutrition. 2000;16(2):120–4.

Wagner D, Adunka C, Kniepeiss D, et al. Serum albumin, subjective global assessment, body mass index and the bioimpedance analysis in the assessment of malnutrition in patients up to 15 years after liver transplantation. Clin Transplant. 2011;25:E396–400.

Part IV

Specific Diseases and Conditions

Biomarkers Associated with Adiposity and Metabolic Dysfunction in Hepatobiliary Tract Cancer

37

Krasimira Aleksandrova, Sabrina Schlesinger, and Marta Stelmach-Mardas

Contents

K. Aleksandrova (✉)
Nutrition, Immunity and Metabolism Start-up Lab, Department of Epidemiology, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany
e-mail: krasimira.aleksandrova@dife.de

S. Schlesinger (✉)
Institut für Epidemiologie, Christian-Albrechts-Universität zu Kiel, Kiel, Germany

Department of Epidemiology and Biostatistics, School of Public Health, Imperial College London, London, UK
e-mail: sabrina.schlesinger@epi.uni-kiel.de; s.schlesinger@imperial.ac.uk

M. Stelmach-Mardas (✉)
Department of Epidemiology, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany

Department of Pediatric Gastroenterology and Metabolic Diseases, Poznan University of Medical Sciences, Poznan, Poland
e-mail: Marta.Stelmach@dife.de

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_37

Abstract

There is ample evidence implicating obesity, nonalcoholic fatty liver disease, and associated metabolic disorders in the risk of hepatobiliary tract cancer. A number of circulating biomarkers related to obesity and metabolic dysfunction could serve as (1) reliable proxies for adiposity-associated disease risk, (2) indicators of intermediate phenotypic alterations, and (3) early markers of elevated disease risk. This book chapter is aimed at providing an overview of recent advances linking biomarkers associated with obesity and impaired metabolism with hepatobiliary tract cancer development and progression. Here, we largely focus on the role of selected metabolic biomarkers – both established and novel ones – as potential intermediates of the association between obesity and liver cancer risk by means of understanding etiology and improving prevention. Overall, evidence has emerged to suggest circulating biomarkers indicative of hyperinsulinemia, biomarkers of chronic low-grade inflammation and immune response, and selected adipose tissue-derived cytokines and hormones to be associated with the risk of the most common form of liver cancer – hepatocellular carcinoma. Moreover, recent evidence largely supports the role of metabolic biomarkers as early disease risk predictors in "low-risk" population groups such as Western Europe and North America. Novel "omics" technologies – metabolomics, proteomics, and glycomics – are intensively being used for the identification of biomarkers in the metabolic pathways. Targeted and untargeted metabolomic approaches have recently led to the discovery of metabolites representing key metabolic alterations in amino acid, polyunsaturated lipid, acetate, and citrate metabolism in the development of liver cancer. Furthermore, metabolic biomarkers were shown to improve primary liver cancer diagnosis beyond the most common biomarkers applied in clinical practice – i.e., alpha-fetoprotein and liver enzyme levels. The role of obesity and metabolic biomarkers was also suggested for gallbladder cancer; however these links remain largely uninvestigated. Despite the given promise for biomarker application, further research is warranted in order to better characterize specific metabolic biomarkers in understanding etiology and their validation as early markers for risk assessment of hepatobiliary tract cancer.

Keywords

Obesity • Nonalcoholic fatty liver disease • Inflammation • Insulin resistance • Metabolic biomarkers • Hepatobiliary tract cancer • Risk prediction • Prevention

List of Abbreviations

BMI	Body mass index
EPIC	European Prospective Investigation into Cancer and Nutrition Cohort
HCC	Hepatocellular carcinoma
HOMA-IR index	Homeostasis model assessment of insulin resistance index
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL-6	Interleukin-6
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
TNF-a	Tumor necrosis factor-alpha

Definitions of Words and Terms

Adipokines	Adipokines also called adipocytokines are a group of hormones secreted by the adipose tissue – around the abdominal area of human body. These molecules are known to have diverse roles, from functions in the individual cell to the whole body. Adipokines have been implicated in a number of diseases and conditions such as nonalcoholic fatty liver disease, metabolic syndrome, type 2 diabetes, heart disease, and recently also to some cancers, including liver cancer.
HCC	Hepatocellular carcinoma (HCC) is a primary form of liver cancer and occurs predominantly in individuals with underlying chronic liver disease and cirrhosis. This is the most common form of liver cancer in adults. About four of five cancers that start in the liver are from this type. HCC can occur and grow in different ways. Some HCCs begin as a single tumor that grows larger. Only late in the disease does it spread to other parts of the liver. A second type can start as many small cancer nodules throughout the liver, not just a single tumor. This is seen most often in people with cirrhosis (chronic liver damage). In this book chapter, the term "liver cancer" is often used to mean hepatocellular carcinoma.

Hyperinsulinemia	Hyperinsulinemia means that the amount of insulin in the blood is higher than considered normal among nondiabetics. Hyperinsulinemia is known to preclude type 2 diabetes. Insulin resistance is the primary cause of hyperinsulinemia, with the pancreas compensating by producing more insulin. This means that when a person has hyperinsulinemia, controlling blood sugar becomes difficult, mostly due to the elaborated function of the pancreas which has to secrete larger amounts of insulin to keep blood sugar at a normal level.
Metabolites	Metabolites are the intermediate products of metabolic reactions catalyzed by various enzymes that naturally occur within cells. This term is usually used to describe small molecules, although broader application is often practiced. Primary metabolites are synthesized by the cell because they are indispensable for their growth. Significant representatives are amino acids, alcohols, vitamins, polyols, organic acids, as well as nucleotides (e.g., inosine-5′-monophosphate and guanosine-5′-monophosphate). Secondary metabolites are compounds produced by an organism that are not required for primary metabolic processes, although they can have important ecologic and other functions. They include drugs, fragrances, flavor, dye, pigments, pesticides, and food additives with applications in agriculture, industry, and pharmaceuticals.
MicroRNAs	MicroRNAs constitute a recently discovered class of noncoding RNAs that play key roles in the regulation of gene expression. Acting at the posttranscriptional level, these fascinating molecules may fine-tune the expression of as much as 30% of all mammalian protein-encoding genes. Mature microRNAs are short, single-stranded RNA molecules approximately 22 nucleotides in length. MicroRNAs are sometimes encoded by multiple loci, some of which are organized in tandemly co-transcribed clusters.
Nonalcoholic fatty liver disease (NAFLD)	NAFLD is the accumulation of extra fat in liver cells that is not caused by alcohol. Despite that normally the liver contains fat, when the fat volume exceeds 5–10% of the liver's weight, then it is called a fatty liver (steatosis).

Introduction

Epidemiology of Primary Liver Cancer

Worldwide, primary liver cancer is the fifth most common cancer in men and the ninth most common cancer in women, representing the second leading cause of cancer-related death worldwide (Torre et al. 2015). In 2012, 782,000 new liver cancer cases were diagnosed and nearly 746,000 deaths, caused by liver cancer, occurred worldwide (Ferlay et al. 2014). Rapid development and early metastasis are the typical characteristics of primary liver cancer, which always results in a poor prognosis. The number of deaths per year in hepatocellular carcinoma (HCC) is virtually identical to the incidence throughout the world, underscoring the high case fatality rate of this aggressive disease. Thus, with a 5-year survival rate between 5% and 9%, primary liver cancer accounts to the most malignant tumors (Jemal et al. 2011). The most common type of primary liver cancer is (HCC), a cancer that develops in hepatocytes. HCC accounts for approximately 85–95% of primary liver cancer, followed by intrahepatic bile duct cancer, a cancer that develops in the bile ducts inside the liver (El-Serag and Rudolph 2007; Torre et al. 2015). The incidence rates of HCC vary a lot across geographical regions, whereas the majority of HCC cases (more than 80%) occurred in less developed countries (El-Serag 2011). The incidence rates are higher in men than in women (Torre et al. 2015). The highest incidence rates for men are detected in Eastern and Southeastern Asia (age-adjusted incidence rate >20 per 100,000), whereas the lowest rates are observed in Northern Europe and South-Central Asia (age-adjusted incidence rate <5 per 100,000) (Fig. 1). For women, the higher incidence rates were detected in Eastern Asia and Western Africa (age-adjusted incidence rate >8 per 100,000) and the lowest in Northern Europe (age-adjusted incidence rate <2 per 100,000) (Torre et al. 2015). However, recent data indicated that HCC rates are also increasing in "lower-risk" Western countries (Center and Jemal 2011).

Risk Factors of Primary Liver Cancer

Most HCC cases arise by reason of an underlying liver disease, which might be partly an explanation for the geographic variation of HCC occurrence (El-Serag 2011). The prevalence of liver cirrhosis in consequence of viral infection with hepatitis B or C, and exposure to toxins, such as aflatoxin or alcohol abuse, is more common in less developed countries compared with Western countries (Cabibbo and Craxi 2010; El-Serag and Rudolph 2007). In contrast, the most common form of liver disease in Western countries includes nonalcoholic fatty liver disease (NAFLD), which is defined by the accumulation of excessive fat in the liver in the absence of alcohol abuse (Table 1). NAFLD includes a spectrum of

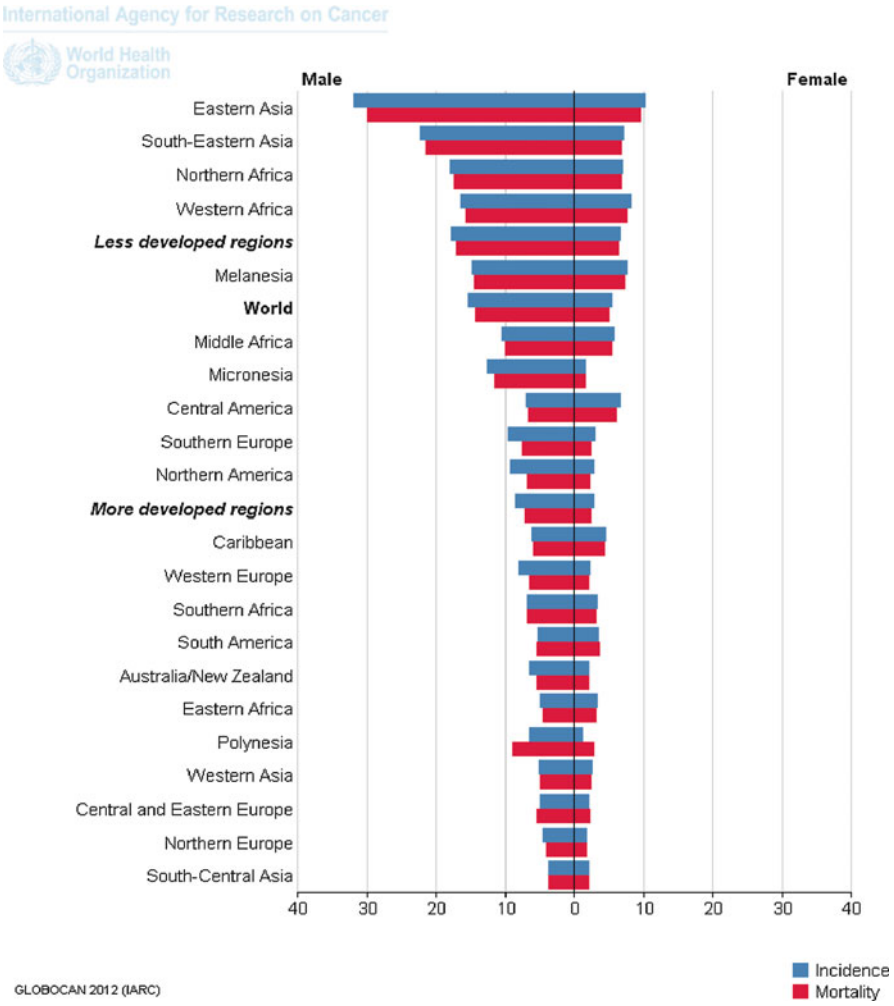

Fig. 1 Estimated age-standardized incidence and mortality rates of liver cancer in 2012, by sex and geographic region. Liver cancer is largely a problem of the less developed regions where 83% (50% in China alone) of the estimated 782,000 new cancer cases worldwide occurred in 2012. It is the fifth most common cancer in men (554,000 cases, 7.5% of the total) and the ninth in women (228,000 cases, 3.4%). Intermediate rates occur in Southern Europe (9.5) and Northern America (9.3) and the lowest rates are in Northern Europe (4.6) and South-Central Asia (3.7). Liver cancer is the second most common cause of death from cancer worldwide, estimated to be responsible for nearly 746,000 deaths in 2012 (9.1% of the total). The prognosis for liver cancer is very poor (overall ratio of mortality to incidence of 0.95), and as such the geographical patterns in incidence and mortality are similar (Data are from Globocan (2012), with permission from the Publishers)

liver disorders, ranging from simple steatosis (infiltration of fat in the liver), to nonalcoholic steatohepatitis (NASH), to cirrhosis, which is related to inflammation, fibrosis, and hepatic injury (Caldwell et al. 2004; Vanni et al. 2010). The

Table 1 Key facts about nonalcoholic fatty liver disease (NAFLD). This table lists the key facts about NAFLD, including its definition, description of the risky phenotypes, its symptoms, diagnosis, and complications to nonalcoholic steatohepatitis (*NASH*) and cirrhosis as pathological prerequisites for the development and progression to liver cancer

NAFLD is the buildup of extra fat in liver cells that is not caused by alcohol. It is normal for the liver to contain some fat. However, if more than 5–10% percent of the liver's weight is fat, then it is called a fatty liver (steatosis)
NAFLD tends to develop in people who are overweight or obese or have diabetes, high cholesterol, or high triglycerides. Rapid weight loss and poor eating habits also may lead to NAFLD. However, some people develop NAFLD even if they do not have any risk factors. NAFLD affects up to 25% of people in the United States
NAFLD may cause the liver to swell (steatohepatitis). A swollen liver may cause scarring (cirrhosis) over time and may even lead to liver cancer or liver failure. What is risky about NAFLD is the lack of evident early symptoms to manifest the disease
In some cases people with NAFLD may experience fatigue, weakness, weight loss, loss of appetite, nausea, abdominal pain, spiderlike blood vessels, yellowing of the skin and eyes (jaundice), itching, fluid buildup and swelling of the legs (edema) and abdomen (ascites), and mental confusion
NAFLD is initially suspected if blood tests show high levels of liver enzymes. However, other liver diseases are first ruled out through additional tests. Often, an ultrasound is used to confirm the NAFLD diagnosis
The more severe form of NAFLD is called NASH. NASH causes the liver to swell and become damaged. NASH tends to develop in people who are overweight or obese or have diabetes, high cholesterol, or high triglycerides. However, some people have NASH even if they do not have any risk factors. Most people with NASH are between the ages of 40 and 60 years. It is more common in women than in men. NASH often has no symptoms and people can have NASH for years before symptoms occur
NASH is one of the leading causes of cirrhosis in adults in the United States. Up to 25% of adults with NASH may have cirrhosis

estimated prevalence of NAFLD is around 20–35% in developed countries, 2–3% of adults are thought to meet current diagnostic criteria for NASH, and eventually up to one third of those with NASH suffer from progressive fibrosis or even cirrhosis (Bellentani and Marino 2009). In obesity defined as BMI above 30 kg/m^2 and in morbidly obese patients, these values are much higher, and patients with NASH are overrepresented in these populations (Fig. 2). It has been suspected that an excess fat storage leads to changes in a number of biological pathways associated with accumulation of fat in the liver, leading to abnormalities in the hepatic metabolism (Eguchi et al. 2006, 2011; Wree et al. 2011). These include oxidative stress (imbalance between prooxidant and antioxidant chemicals that lead to liver cell damage); production and release of toxic inflammatory proteins (cytokines) by the patient's own inflammatory cells, liver cells, or fat cells; liver cell necrosis or death, called apoptosis; adipose tissue (fat tissue) inflammation and infiltration by white blood cells; and disrupted balance in gut microbiota (intestinal bacteria) which may play a role in liver inflammation (Fig. 3; Buechler et al. 2011). In particular, fat accumulation in the abdominal, intra-abdominal (or visceral), and hepatic depots has been associated with elevated risk of metabolic diseases. The

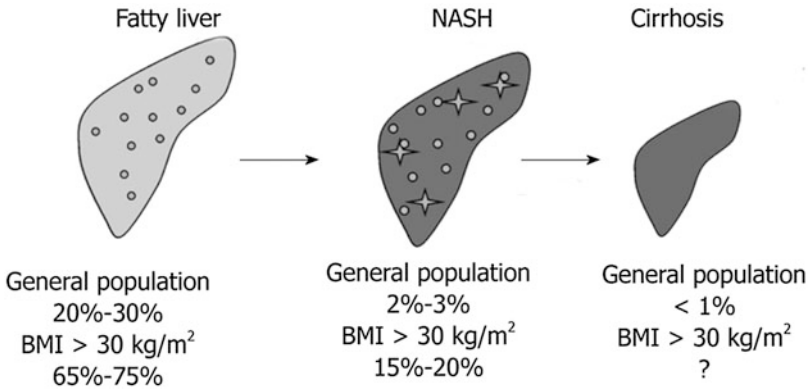

Fig. 2 Prevalence of fatty liver, (NASH), and obesity-related liver cirrhosis in the general population. Current estimates based on different studies in unselected and selected populations indicate that about 20–30% of adults in Western countries have excess fat accumulation in the liver, 2–3% of adults are thought to meet current diagnostic criteria for NASH, and eventually up to one third of those with NASH suffer from progressive fibrosis or even cirrhosis. In obesity defined as BMI above 30 kg/m^2 and in morbidly obese patients, these values are much higher and patients with NASH are overrepresented in these populations (Data are from Buechler et al. (2010), with permission from the Publishers)

adipose tissue itself is defined as an endocrine organ that produces several hormones and proteins, including growth factors, inflammatory biomarkers, and adipocytokines, which are involved in metabolic distortions and diseases, which may promote risk of cancer, including HCC (Calle and Kaaks 2004; Greenberg and Obin 2006).

Potential Applications to Prognosis of Primary Liver Cancer

The high malignancy of liver cancer coupled with its late diagnosis and consequent poor survival could be attributed to its asymptomatic manifestation at early stages. Therefore, improving both the understanding of HCC etiology and the early detection of the disease is an important first step toward the design of effective prevention strategies. In this regard, the identification of novel metabolic biomarkers as early predictors of risk of liver cancer could be a step forward for early detection, diagnosis, and assessment of applied medical therapies to cancer prevention and treatment (Roessner et al. 2001). Parallel advancements in the complementing fields of biology, medicine, and genetics induced by boosting technological developments have led to numerous novel biomarker discoveries linking metabolism and cancer.

Herewith, we reviewed recent advances on biomarkers associated with obesity and impaired metabolism and the risk of hepatobiliary tract cancer development and

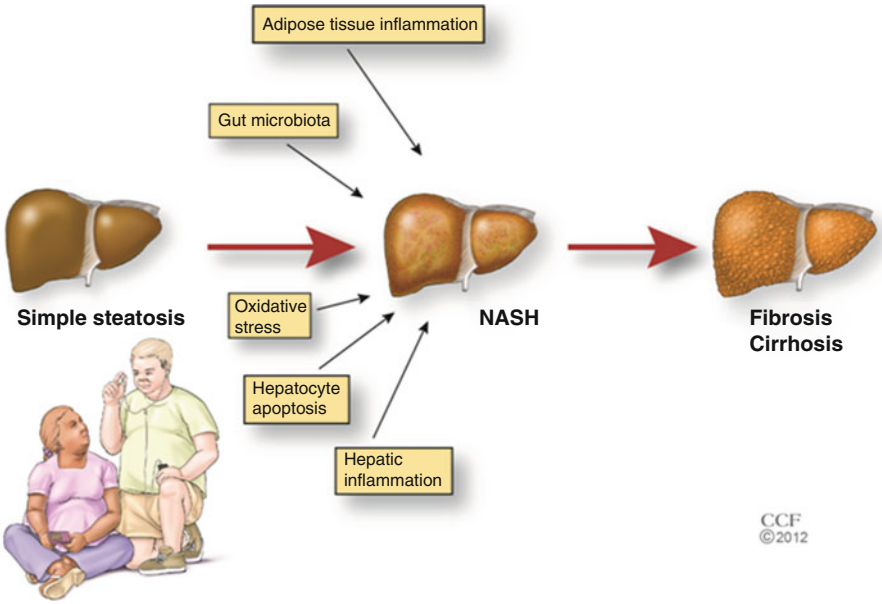

Fig. 3 Factors that may contribute to the development of nonalcoholic fatty liver disease (NASH) and hepatic fibrosis and cirrhosis. The figure outlines specific factors of relevance to the development of NASH and hepatic fibrosis and cirrhosis. Excess fat storage leads to changes in a number of biological pathways associated with accumulation of fat in the liver, leading to abnormalities in the hepatic metabolism. These include oxidative stress (imbalance between prooxidant and antioxidant chemicals that lead to liver cell damage); production and release of toxic inflammatory proteins (cytokines) by the patient's own inflammatory cells, liver cells, or fat cells; liver cell necrosis or death, called apoptosis; adipose tissue (fat tissue) inflammation and infiltration by white blood cells; and disrupted balance in gut microbiota (intestinal bacteria) which may play a role in liver inflammation (Adapted from Naim Alkhouri et al. 2012)

progression. The review provides a special focus on the role of selected metabolic biomarkers – both established and novel ones – as potential intermediates of the association between obesity and liver cancer by means of understanding etiology and improving prevention.

Obesity and Hepatobiliary Cancer

Recently, the World Cancer Research Fund concluded that there is a strong evidence to establish body fatness as an important risk factor for liver cancer. The reviewed evidence was exclusively based on studies investigating body fatness defined by measurements of body mass index (BMI: weight/height^2 [kg/m^2]) as an anthropometric indicator of general overweight and obesity (Fig. 4). Despite BMI being a good marker for total body fatness, it does not

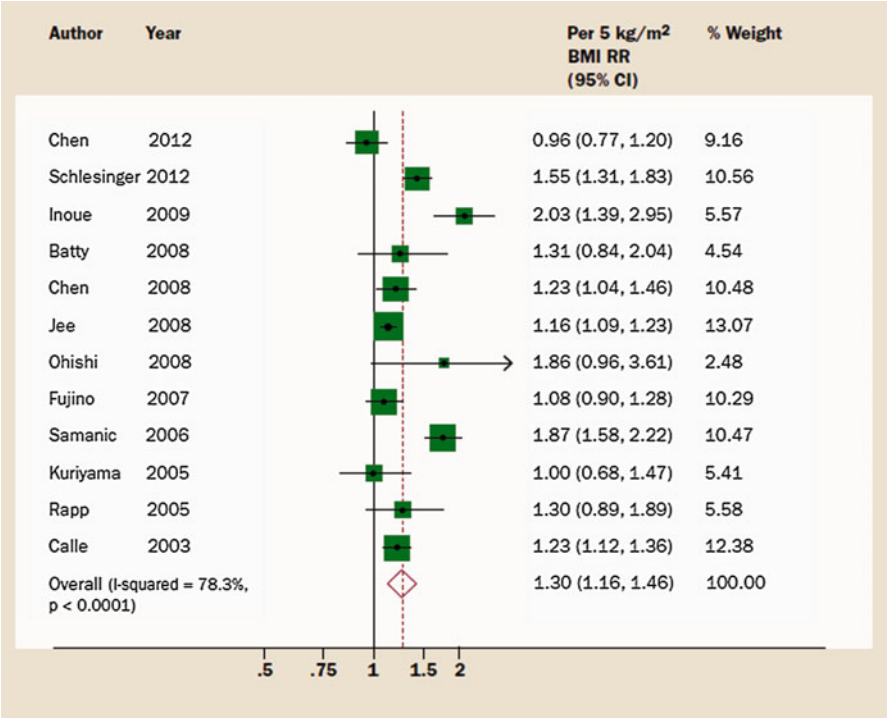

Fig. 4 Association between obesity based on BMI measurements and risk of liver. The World Cancer Research Fund Report identified 15 studies on liver cancer. Of 11 studies (13 estimates) reporting on liver cancer incidence, nine reported a positive association when comparing the highest and the lowest categories, of which six were statistically significant. Twelve of 15 studies on liver cancer were included in the dose–response meta-analysis ($n = 14{,}311$), which showed a statistically significant increased risk of 30% per 5 kg/m2 [RR 1.30 (95% CI 1.16–1.46]. There was evidence of nonlinearity ($p < 0.0001$), with a steeper increase in risk at higher BMI levels. A dose–response meta-analysis showed an increased risk per 5 kg/m^2 for both liver cancer incidence and mortality. There was a statistically significant increased risk per 5 kg/m^2 for both men and women. When stratified by geographical location, dose–response meta-analyses showed a statistically significant increased risk per 5 kg/m^2 in both European and Asian studies, with a stronger association in European studies (Data are from World Cancer Research Fund International/American Institute for Cancer Research. Continuous Update Project Report: Diet, Nutrition, Physical Activity and Liver Cancer. 2015), with permission from the Publishers)

account for body fat distribution. Therefore, measures of abdominal obesity might better reflect differences in body shape and fat distribution. The waist circumference and the waist-to-hip and the waist-to-height ratio are established measurements for abdominal obesity. In this context, a large European multicenter study within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort particularly explored the association between abdominal obesity and risk of HCC (Schlesinger et al. 2013). Abdominal obesity was defined using established cut off values provided by the World Health Organization (waist

circumference ≥102 cm for men and ≥88 cm for women and waist-to-hip ratio ≥95 for men and ≥0.80 for women). The study reported a twofold times higher risk of HCC for abdominally obese individuals compared with nonobese controlling for other factors, such as age, sex, alcohol consumption, smoking status, education, hepatitis B and C virus infection, and even after accounting for general obesity (defined based on BMI measurements). These findings largely support the role of abdominal fat distribution as an independent risk factor for HCC regardless of general obesity (Schlesinger et al. 2013). Anthropometric measures, such as BMI, waist circumference, or waist-hip ratio, have been used as surrogate markers for total and abdominal adiposity: however, their correlations with fat mass could vary by sex, ethnicity, life stages, and other as yet-unknown factors which represent a limitation when studying disease risks. Recently, it has been shown that these anthropometric measures are poorly correlated with fat compartments involved in metabolic risk, such as visceral and hepatic fat. These recognized limitations of anthropometric indicators have recently provoked an enlarged interest in utilizing biomarkers assessed in peripheral blood as an alternative to, or complement, anthropometry as predictors of body fat composition and distribution (Lim et al. 2012; van Dijk et al. 2010). Furthermore, circulating biomarkers may represent intermediate pathways that may help in better understanding of the link between obesity, NAFLD, and metabolic dysfunction with liver cancer development and progression (Caldwell et al. 2004; Marchesini et al. 2008).

Circulating Biomarkers of Hyperinsulinemia

One of the most implicated mechanisms linking obesity-related metabolic diseases and liver cancer risk is insulin resistance and, in particular, hyperinsulinemia in the setting of chronic liver disease. Insulin resistance is defined as a refractory state of the liver to the negative regulatory effect of insulin on glucose production. Rise in circulating insulin levels is frequently associated with chronic liver disease, resulting both from the impairment of hepatic insulin degradation and from the activation of insulin secretion by pancreatic beta-cells, a compensatory mechanism allowing to maintain euglycemia in the early course of insulin-resistant states (Chettouh et al. 2015). Accumulating evidence suggests that there is a causative link between hyperinsulinemia and HCC development and progression (Chettouh et al. 2015). Hyperinsulinemia may affect liver cancer development and progression not only through direct effects on the growth of preneoplastic and transformed hepatocytes but also indirectly by increasing the production of cytokines and mitogens, enhancing fibrosis and promoting angiogenesis. While the activation of insulin-dependent signaling pathways is certainly not sufficient to initiate tumorigenesis on its own, hepatocytes may develop adaptive insulin-dependent mechanisms to proliferate and survive during chronic liver disease which may in turn promote premalignant transformation and tumor growth. In addition, insulin may foster a microenvironment milieu favorable to the propagation of premalignant and malignant cells.

Circulating biomarkers indicative of hyperinsulinemia include insulin-like growth factors I and II (IGF-I and IGF-II), their receptors and their binding proteins (LeRoith et al. 1995), as well as C-peptide and homeostasis model assessment of insulin resistance (HOMA-IR) index.

Biomarkers in the IGF-I/IGFBP Pathway

The IGF-I is a well-known mitogen that is primarily produced in the liver and shares sequence homology with insulin. Within circulation and tissue compartments, IGF is bound with high affinity to a family of structurally related binding proteins (IGFBP) characterized by different properties (Clemmons 1997). In rat models of hepatocarcinogenesis, increased expressions of IGF-I and IGF-binding protein-4 (IGFBP-4) in altered parenchymal cells and a decreased expression of IGFBP-1 have been demonstrated (Scharf et al. 2000). Chronic hyperinsulinemia may lead to increase circulating levels of free and bioactive IGF-I due to stimulation by insulin growth hormone receptor expression or decreased hepatic synthesis and blood levels of IGF-binding proteins (IGFBP)-1 and IGFBP-2. Paradoxically, a significant inverse association has been found between IGF-I and insulin levels in patients with HCC in the setting of chronic liver diseases, which poses the hypothesis that hyperinsulinemia is associated with higher levels of IGF-I at early but not advanced disease stage (Hung et al. 2014). Indeed, IGF-I serum levels have been found to decrease gradually when compared between healthy subjects, patients with cirrhosis and patients with HCC. The IGF-I deficiency in HCC is thought to result from the reduced synthesis capacity of the cirrhotic liver mass (Chettouh et al. 2015). The inverse association could be also explained by blocking of the IGF-I production by selected cytokines, including tumor necrosis factor-alpha (TNF-a) and interleukin-6 (IL-6), highly expressed in patients with HCC (Hung et al. 2014).

Biomarkers of Systemic Insulin Resistance

The HOMA-IR index is an accepted marker of systemic insulin resistance which integrates both fasting glycemia and insulinemia. It is calculated by the following formula: HOMA-IR index =fasting glucose (mmol/L) x fasting insulin (mIU/L)/ 22.5. The HOMA-IR index has been associated with HCC development and liver-related death in hepatitis C patients with cirrhosis, independently of BMI (Nkontchou et al. 2010) or regardless of the presence of diabetes (Chao et al. 2011). In particular, elevated fasting insulin has been shown to be an independent risk factor for HCC in a 17-year follow-up of a population-based prospective cohort of 2,903 male hepatitis B carriers (Chao et al. 2011). More recently, baseline serum levels of C-peptide – a circulating biomarker released from proinsulin during beta-cell insulin secretion – have been found to be associated with a higher risk of HCC in the prospective EPIC cohort, independent of obesity parameters and other

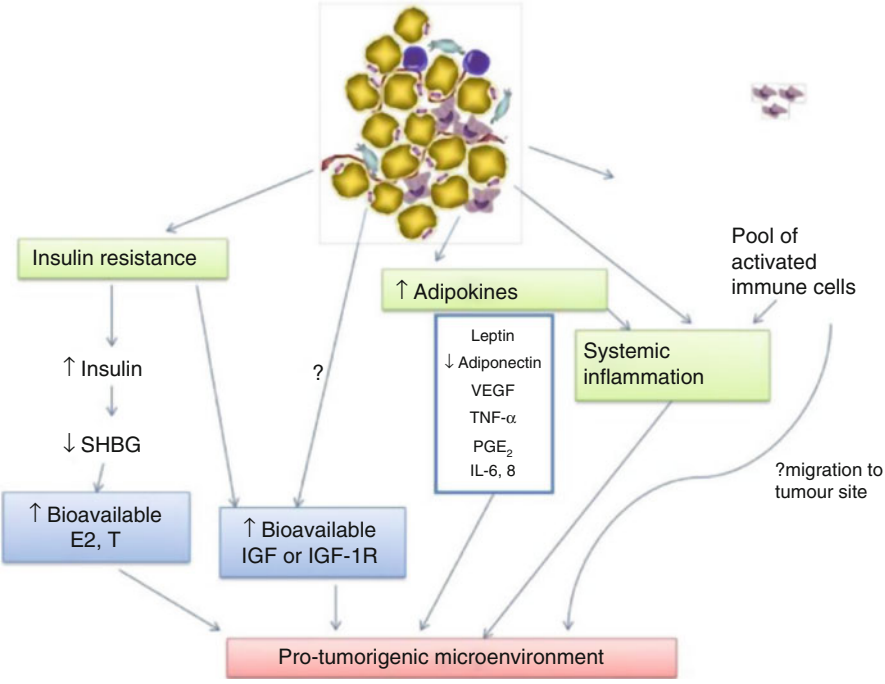

Fig. 5 Systemic alterations in visceral adiposity which may contribute to a pro-tumorigenic microenvironment. Systemic alterations in obesity include chronic systemic inflammation, increased adipokine production, and an altered immunological status. Additionally, there are associated changes in the sex hormone profile. Insulin resistance develops as a consequence of visceral adiposity and there is a rise in insulin production, which may be associated with activation of the insulin-like growth factor (*IGF*) system. All of these changes which occur in tandem with the development of obesity have the ability to interact with each other. It is this altered systemic milieu which is thought to fuel cancer development and progression (Data are from Donohoe et al. (2011), with permission from the Publishers)

established liver cancer risk factors, including among others hepatitis B and C virus infection and inflammatory markers (Aleksandrova et al. 2014; Fig. 5).

Biomarkers of Liver Fat Accumulation

Emerging evidence has suggested fetuin-A as a proxy biomarker of liver fat accumulation and associated metabolic consequences. Fetuin-A is a plasma protein exclusively secreted by the liver in humans, known to be upregulated in liver dysfunction and to correlate with key enzymes in glucose and lipid metabolism (von Loeffelholz et al. 2016). Population studies suggested that fetuin-A levels are elevated in NAFLD and its hepatic expression of fetuin-A correlates with key enzymes in glucose and lipid metabolism (Haukeland et al. 2012). As fetuin-A has been suggested to provide a link between fatty liver and insulin resistance, it could

be implicated in the development of liver cancer. The only prospective investigation of pre-diagnostic circulating levels of fetuin-A within the EPIC cohort showed no significant association of fetuin-A with HCC risk (Aleksandrova et al. 2014).

Biomarkers of Obesity-Induced Liver Inflammation

Obesity is related with pro-inflammatory molecules – chemokines and cytokines – involved the initiation and progression of HCC (Marra and Tacke 2014; Qiao and Li 2014). In obese patients, accumulation of lipids in the liver promoted activation of an inflammatory response. At the same time, lipid accumulation increases demand on the endoplasmic reticulum leading to uncontrolled production of reactive oxygen species. Oxidative stress stimulates further inflammatory signaling and induces oxidative damage including strand breaks and nucleotide modifications, and DNA damage leading to genomic instability. Chemokines and their receptors can largely contribute to the pathogenesis of HCC, promoting proliferation of cancer cells, the inflammatory microenvironment of the tumor, evasion of the immune response, and angiogenesis (Marra and Tacke 2014). Although acute liver inflammation can play a vital and beneficial role in response to liver damage or acute infection, the effects of chronic liver inflammation, including liver fibrosis and cirrhosis, are sufficient in a fraction of individuals to initiate the process of transformation and the development of HCC (Stauffer et al. 2012). Sustained hepatic inflammation results in damage to parenchyma, oxidative stress, and compensatory regeneration/proliferation. Chronic inflammation is associated with persistent liver injury and consecutive regeneration, potentially leading to fibrosis and cirrhosis and, consequently, to the development of HCC. Chronic inflammation may also originate from hepatotropic viruses, toxins, or impaired autoimmunity. Mechanisms that link inflammation and liver cancer are not completely understood, but transcription factors of the nuclear factor kappa B family and signal transducer and activator of transcription 3, cytokines such as IL-6, and ligands of the epidermal growth factor receptor family are pivotal players. Chronic inflammation is associated with persistent liver injury and consecutive regeneration, potentially leading to fibrosis and cirrhosis and, consequently, to the development of HCC; however evidence remains scarce. Recently, data from the EPIC cohort provided first lines of evidence for an independent association between several inflammatory and metabolic biomarkers and HCC risk suggesting their role as intermediate factors in the obesity-liver cancer association (Aleksandrova et al. 2014). These biomarkers improved risk assessment of HCC beyond established risk factors such as hepatitis infection, smoking, alcohol consumption, and beyond alpha-fetoprotein levels (Fig. 6). These data has been confirmed by a recent case–control study nested in a Japanese cohort with 188 HCC cases and 605 controls which reported that higher concentrations of CRP and Il-6 have been associated with an around twofold and fivefold higher risk of liver cancer, respectively (Ohishi et al. 2014). These associations were independent of hepatitis virus infection, lifestyle-related factors, and radiation exposure. The fact that observed associations were independent of established HCC risk factors and adiposity measures largely suggests an intermediary role of these

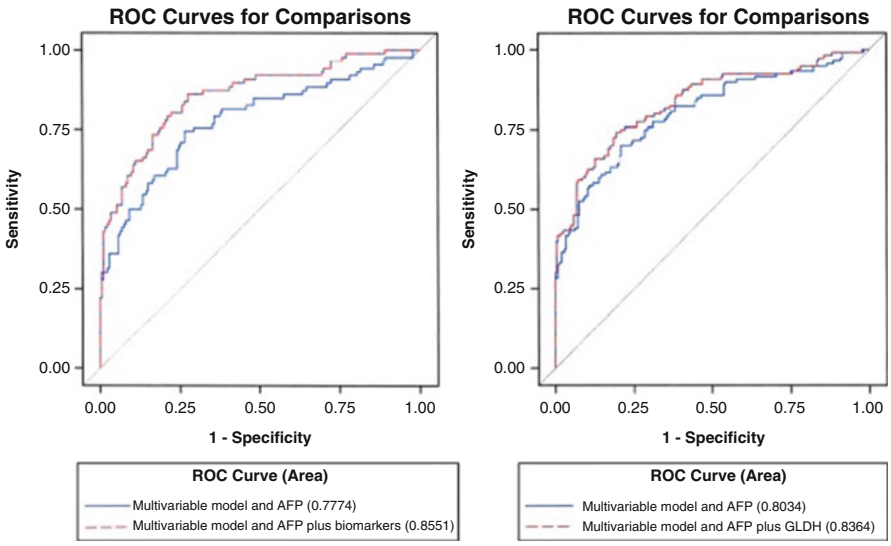

Fig. 6 Predictive ability of inflammatory and metabolic biomarkers beyond established liver cancer risk factors. The figure represents the predictive ability of inflammatory and metabolic biomarkers beyond the multivariable-adjusted model and alpha-fetoprotein (*AFP*) levels in a nested case–control study within the EPIC cohort among 125 HCC cases and 250 matched controls. The biomarkers included in the model have been associated with HCC risk. These include CRP, Il-6, C-peptide, and non-HMW adiponectin. Multivariable model taking into account matching factors: study center, gender, age (±12 months), date (±2 months), fasting status (<3, 3–6, or >6 h), and time of the day (±3 h) at blood collection. Women were additionally matched according to menopausal status (pre-, peri- [unknown], or postmenopausal) and exogenous hormone use (yes, no, or missing) at blood donation. Further adjusted for education (no school degree or primary school, secondary school, high school, or missing), smoking (never, former, current, or missing), alcohol at baseline, drinking status at baseline (nondrinker or drinker), diabetes (no, yes, or missing), coffee (g/day), HBsAg/anti-HCV (negative, positive, or missing), BMI, and WHtR adjusted for BMI (Data are from Aleksandrova et al. (2014), Open Access Article, available under the terms of the Creative Commons Attribution Non-Commercial No Derivatives License CC BY-NC-ND)

biomarkers on the causal pathway between obesity and HCC risk (Aleksandrova et al. 2014). However, exact mediating roles of metabolic biomarkers on the association between obesity and HCC are not established so far.

Biomarkers Related to Excess Adipose Tissue Production

Adipose tissue is increasingly recognized as an endocrine organ secreting a variety of biologically active molecules, known as "adipokines." Adipokines play an important role in the physiology of adipose tissue, including food intake and nutrient metabolism, insulin resistance, oxidative stress, low-grade inflammation, and immunity. Several studies reported that adipokine dysregulation contribute to liver fibrosis and influence the pathological state of chronic liver diseases (Abenavoli and Peta

2014; Bekaert et al. 2016; Bertolani and Marra 2008; Kalafateli et al. 2015; Polyzos et al. 2015; Stojsavljevic et al. 2014). The dysregulated expression of adipokines may therefore provide explanatory mechanisms in the association of obesity with HCC (Jung and Choi 2014). Among various adipokines, two molecules – leptin and adiponectin – gained much attention in the recent research.

Leptin

Leptin, the product of the (ob) gene, is a well-known pleiotropic adipokine best known as a regulator of food intake and energy expenditure via hypothalamic-mediated effects. Leptin is closely linked with the higher BMI and thereby considered as a good proxy measure of general adiposity (Shah and Braverman 2012). Leptin increases with increasing fatty mass as a compensatory mechanism to preserve insulin sensitivity, but persistent hyperleptinemia could be implicated in liver fibrinogenesis and carcinogenesis (Dutta et al. 2012; Polyzos et al. 2011). Leptin plays profibrogenic and pro-angiogenic roles by acting on hepatic stellate cells and Kupffer cells to synthesize extracellular matrix components, pro-inflammatory or pro-angiogenic cytokines. Leptin could play a role in the development of NAFLD through insulin resistance, steatosis, worsening hepatic inflammation, and ultimately fibrosis; however its role during the development of chronic kidney disease and cancer development and progression is not well understood and the evidence remains inconclusive. A recent meta-analysis of 33 studies among 2,612 individuals concluded that circulating leptin levels were higher in patients with NAFLD than in controls (Polyzos et al. 2016). Higher levels of circulating leptin were associated with increased severity of NAFLD, and the association remained significant after exclusion of studies involving adolescent populations and morbidly obese individuals (Polyzos et al. 2016). Leptin has angiogenic properties, promotes cell proliferation and migration, and interacts with growth factors, all of which could promote tumor growth (Dutta et al. 2012). However the role of leptin in the development of liver cancer remains controversial with some studies suggesting an important role of leptin in liver fibrosis and carcinogenesis (Ribatti et al. 2008), while others demonstrating an inhibitory role of exogenous leptin on tumor size in murine model of HCC (Elinav et al. 2006). So far, the association of leptin and liver cancer was explored in only one prospective epidemiological study – the EPIC cohort – which suggested a null association (Aleksandrova et al. 2014).

Adiponectin

Adiponectin is one of the most abundantly secreted adipokines in blood circulation, which actions are mainly exerted by the activation of AMP-activated kinase and peroxisome proliferator-activated receptor alpha (Moschen et al. 2012). The liver is a major target organ of adiponectin metabolism (Moschen et al. 2012). Adiponectin is implicated in the regulation of steatosis, insulin resistance, inflammation, and fibrosis;

thereby it could be expected that hyperadiponectinemia might suppress liver tumorigenesis and elevated levels of adiponectin would be associated with a reduced risk of HCC (Wieser et al. 2012). In contrast, experimental studies indicated that adiponectin treatment increased apoptosis of HCC and inhibited its proliferation (Kamada et al. 2008; Wieser et al. 2012). Some studies have shown that circulating adiponectin levels are higher in subjects with liver cirrhosis and that they increase in line with fibrosis stage (Nkontchou et al. 2010). Paradoxically, several human studies suggested that elevated adiponectin concentrations are positively associated with HCC risk. A nested case–control study conducted in middle-aged Japanese adults with hepatitis virus infection showed that both total and high-molecular-weight adiponectin are associated with a higher risk of HCC (Michikawa et al. 2013). A hospital-based cohort study in Japan showed that high serum levels of adiponectin were positively associated with the development of HCC in patients with chronic hepatitis C infection (Arano et al. 2011). In line with these studies, in the large sample of the EPIC cohort, higher levels of adiponectin and in particular its non-high-molecular-weight isoform were associated with a lower risk of HCC risk (Aleksandrova et al. 2014). A possible explanation for the positive associations between adiponectin and HCC risks could be sought in the typically observed liver function impairment in liver diseases (including cirrhosis) leading to induced adiponectin production (hyperadiponectinemia). However, the role of adiponectin was not supported by all studies such that in a recent French study comprising of 248 patients with compensated cirrhosis null findings have been reported (Nkontchou et al. 2010).

Chemerin, Resistin, and Adipocyte-Fatty-Acid-Binding Protein

Apart from established adipokines, such as leptin and adiponectin, a recent systematic review evaluated the potential link between newly described adipokines and liver histology in biopsy-proven NAFLD patients (Bekaert et al. 2016). Thirty-one cross-sectional studies were included, resulting in a total of seven different investigated adipokines, most of which suggested to be involved in the inflammatory response that develops within the context of NAFLD, either at hepatic or systemic level, and/or hepatic insulin resistance. Based on this literature, review clinical studies suggest that chemerin, resistin, and adipocyte-fatty-acid-binding protein potentially are involved in NAFLD pathogenesis and/or progression (Bekaert et al. 2016). However, major inconsistency still exists, and there is a high need for larger studies using standardized assays to determine adipokine levels.

Novel Biomarker Discoveries: Metabolomics, Proteomics, and Glycomics

Since HCC development implies alterations in the metabolic functions of the liver and, in a majority of cases, progresses from precancerous lesions through to cirrhosis and cancer, it is conceivable that metabolic changes may be detected from the very

early stages of the disease, long prior to clinical diagnosis. New techniques such as metabolomics, proteomics, and glycomics may not only allow development of novel markers but also allow us a better insight into the metabolic pathophysiology of HCC (Fitzpatrick and Dhawan 2014).

Biomarkers in Metabolomics

Metabolomics is the latest of the omics technologies that employs state-of-the-art analytical instrumentation in conjunction with pattern recognition techniques to discover metabolic changes in subjects related to disease status. Thus, metabolomics may serve as a valuable tool for the identification of biomarkers for early detection of HCC. The metabolic analysis of patients with NAFLD showed higher levels of glycocholate, taurocholate, and glycochenodeoxycholate (Kalhan et al. 2011). Additionally, plasma concentrations of long-chain fatty acids were lower, and concentrations of free carnitine, butyrylcarnitine, and methylbutyryl carnitine were higher in NASH. Several glutamyl dipeptides were higher, while cysteine–glutathione levels were lower in NASH and steatosis. Other changes included higher branched-chain amino acids, phosphocholine, carbohydrates (glucose, mannose), lactate, pyruvate, and several unknown metabolites. Further, it has been also found that the combination of glucose, lactate, glutamate/glutamine, and taurine can be indicative in the risk of NAFLD progression (Li et al. 2011). Serum and urine metabolite markers relevant to the HCC are mostly involved in metabolism of bile acids, free fatty acids, urea cycle, and methionine. Some of them (bile acids, histidine, inosine) are promising and need a validation as a single biomarker for HCC. Others, such as several bile acids, cholic acid, glycocholic acid, deoxycholic acid, and glycochenodeoxycholic acid, can be used in the stratification of HCC subjects with and without cirrhosis and hepatitis. A recent report characterized the metabolic features of HCC using a nontargeted metabolic profiling strategy based on liquid chromatography-mass spectrometry (Huang et al. 2013). Fifty pairs of liver cancer and matched normal tissues were collected from HCC patients, including tumor tissues, adjacent noncancerous tissues, and distal noncancerous tissues, and 105 metabolites were filtered and identified from the tissue metabolome. The principal metabolic alternations in HCC tumors included elevated glycolysis, gluconeogenesis, and β-oxidation with reduced tricarboxylic acid cycle and Δ-12 desaturase. Furthermore, increased levels of glutathione and other anti-oxidative molecules, along with decreased levels of inflammatory-related polyunsaturated fatty acids and phospholipase A2, were observed. The diagnosis potential of the differential metabolites found in tissues, further studied in serum samples, and validated in another group of serum samples, demonstrated that the combination of betaine and propionylcarnitine has a better prediction than AFP; it is useful for both AFP false-positive and false-negative patients in distinguishing HCC from hepatitis and cirrhosis. External validation of cirrhosis and HCC serum specimens further showed that this combination biomarker is useful for HCC diagnosis with a supplementary role to alpha-fetoprotein. Although a number of metabolomic-

based studies have been applied to HCC, they have either been largely based on traditional case–control designs; high risk patient groups characterized by hepatitis infection, cirrhosis, or other chronic liver diseases; and non-Western populations characterized by high prevalence of traditional risk factors – hepatitis infection and aflatoxin exposure. Collectively, those studies reported a potential impairment of the tricarboxylic acid cycle, increased lipid catabolism, elevation of essential amino acids, and defects on ammonium detoxification and increased fatty acid beta-oxidation in HCC. However, there is currently very little information derived from prospective settings where biological samples have been collected prior to disease diagnosis. Recently, data from a large prospective EPIC cohort provided first insights on the prospective association of metabolomic biomarkers and risk of hepatobiliary tract cancer, such that metabolomic profiles could likely indicate pre-diagnostic changes in cancer-free cohort participants. In a nested case–control study within the EPIC cohort circulating levels of amino acids (AA) and their derivatives, biogenic amines and hexoses were measured using targeted metabolomics approach (Biocrates AbsoluteIDQ p180 Kit) and their association with HCC and other anatomically related cancers of the intrahepatic bile duct, and gallbladder and extrahepatic biliary tract was evaluated (Stepien et al. 2016). The study identified fourteen metabolites to be significantly associated with HCC risk. Among associated metabolites, leucine, lysine, glutamine, and the ratio of branched chain to aromatic AA were inversely, while phenylalanine, tyrosine and their ratio, glutamate, glutamate/glutamine ratio, kynurenine, and its ratio to tryptophan were positively associated with HCC risk. However, the study detected a strong confounding effect by hepatitis status and liver enzyme levels. In another recent study within the EPIC cohort that applied an untargeted nuclear magnetic resonance metabolomic approach, sixteen individual metabolites representing key metabolic alterations in amino acid, polyunsaturated lipid, acetate, and citrate metabolism were found to be significantly associated with HCC risk. Importantly, this study showed that consideration of metabolomic profiles can improve HCC diagnosis beyond that provided by alpha-fetoprotein and liver enzyme levels, which are currently the most common HCC biomarkers in clinical practice (Fig. 7). These associations were suggested to be specific for HCC as analogous study conducted in parallel on extrahepatic/intrahepatic bile duct carcinomas did not reveal significant associations.

Biomarkers in Proteomics

Recently, the identification of protein expression pattern that differs significantly between individuals without fatty liver disease and patients with various forms of NAFLD was prioritized. Consequently, a panel of the following proteins, fibrinogen β-chain, retinol binding protein 4, serum amyloid P component, lumican, transgelin 2, and CD5 antigen-like differentiated groups of patients with simple steatosis, NASH, and NASH F3/F4, was recently established (Bell et al. 2010). Further, complement component C7, insulin-like growth factor acid labile subunit, and transgelin 2 correctly

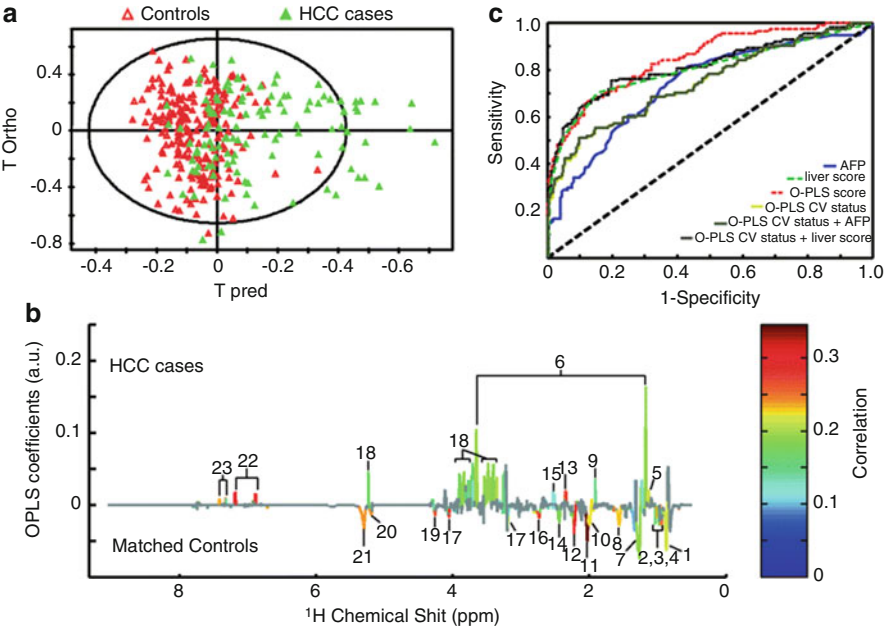

Fig. 7 Nontargeted metabolomic discrimination between liver cancer cases and controls. The figure represents results from untargeted metabolomic analyses among HCC cases ($n = 114$) and matched controls ($n = 222$) in the EPIC cohort study. The analysis is based on H Carr–Purcell–Meiboom–Gill NMR data. (**a**) Orthogonal partial least-square (*O-PLS*) score plot of NMR spectra, $R^2 = 35\%$, $Q^2 = 21\%$. (**b**) O-PLS metabolic signature colored according to the correlation between NMR variables and case–control status after significance to ANOVA tests followed by Benjamini–Hochberg multiple correction (nonsignificant NMR variables are colored in *gray*). The validation of the model is presented in Additional file 1: Figure S1a. *1* $C\mathbf{H}_3$ bond of lipids mainly very-low-density lipoproteins, *2* leucine, *3* isoleucine, *4* valine, *5* propylene glycol, *6* ethanol, *7* $C\mathbf{H}_2$ bond of lipids, *8* $C\mathbf{H}_2$–CH_2–COOC bond of lipids, *9* acetate, *10* $C\mathbf{H}_2$–CH = bond of lipids, *11* *N*-acetyl glycoproteins, *12* acetone and CH_2–$C\mathbf{H}_2$–COOC bond of lipids, *13* glutamate, *14* glutamine, *15* citrate, *16* =CH–$C\mathbf{H}_2$–CH = bond of lipids, *17* choline, *18* glucose, *19* lipid O–$C\mathbf{H}_2$, *20* mannose and lipids, *21* C**H** = C**H** bond of lipids, *22* tyrosine, *23* phenylalanine. An equivalent metabolic signature obtained from ^{1}H NOESY NMR data is presented in Additional file 1: Figure S1b. (**c**) ROC analyses including AFP, liver function score, O-PLS score, O-PLS cross-validated (*CV*) status, and a combination between O-PLS CV status and AFP or liver function score. The ROC of O-PLS CV status and the combination of O-PLS CV status and AFP overlap (Data are from Fages et al. (2015), with permission from the Publishers)

categorized patients as having NAFLD (simple steatosis and NASH) or NASH F3/F4. Finally, prothrombin fragment and paraoxonase 1 were able to accurately differentiate between control group and study patients with all forms of NAFLD. Based on the available literature, patients with NASH seem to have a much higher risk of HCC than patients with simple steatosis. It has been also suggested that cytokeratin-18 fragment (CK-18) can be the most accurate biomarker for NAFLD and NASH patients, which being used together with fibroblast growth factor 21 (FGF21) may improve the accuracy in diagnosing NASH (Shen et al. 2012). Moreover, other studies confirmed that in the

proteomic analysis of obesity-induced mouse livers, changes in cytokeratins (CK-8 and CK-18) and vimentin are linked to hepatic steatosis leading to NASH (Park et al. 2011). The following 6,944 initial NAFLD patients up for 3 years indicated, based on proteomics, that higher baseline hemoglobin values were associated with higher incidence of NAFLD (Yu et al. 2012).

Biomarkers in Glycomics

The arising interest of glycomics is related to the fact that glycosylation is associated with a posttranslational modification of many secreted proteins and structural changes in the glycan structures of serum proteins being an indication for liver damage (Fitzpatrick and Dhawan 2014). It has been suggested that the serum *N*-glycan profile is a promising noninvasive method for detecting NASH or NASH-related fibrosis in NAFLD patients, which could be a valuable supplement to other markers currently used in diagnosis of NASH (Chen et al. 2009). Liver is directly related to the production of most glycoproteins in serum; therefore, the *N*-glycome profile can reflect changes in either the liver or B cell function (Fitzpatrick and Dhawan 2014). Nevertheless, the investigations on serum *N*-glycans that could be used in early detection of HCC development still have to be explored. Recently it has been discovered that G3560 and G2890 (serum *N*-glycans) can be a significant predictors of overall survival and disease-free survival in patients suffering from HCC characterized by high sensitivity and specificity (Kamiyama et al. 2013).

MicroRNAs

MicroRNAs (miRNAs) – a class of small, single-stranded, noncoding RNA molecules which regulate gene expression – might have oncogenic or tumor-suppressive properties in human cancer and have been shown to be involved in carcinogenesis of various cancers. Recently, miRNAs have been recognized as potential biomarkers for NAFLD progression and HCC development. It has been shown that miRNA being endogenous 19–24 nucleotides noncoding single-stranded RNAs can control at the posttranscriptional level complementary target mRNAs implicated in selected pathophysiological process, including development of NAFLD and HCC (Sayed and Abdellatif 2011). Especially miRNAs that are expressed in serum or plasma are characterized as noninvasive for early detection of HCC. MiRNAs are produced as a pre-miRNA and exported from the nucleus to the cytoplasm where they are cleaved by the endoribonuclease called Dicer. During this process, the cleaved portions are unwound to single strands and loaded into the RNA-induced silencing complex (RISC). This miRNA–RISC complex can interact with the 30-end untranslated region (30UTR) of the target gene's messenger RNA resulting in the suppression of mRNA translation or direct degradation of target mRNA (Vincent and Sanyal 2014). It has been also suggested that differentially expressed miRNAs in humans and animal models of NASH regulate genes with diverse functions involved in the

pathogenesis of NAFLD, including metabolism of lipid and glucose, regulation of the unfolded protein response, endoplasmic reticulum stress, oxidative stress, cellular differentiation, inflammation, and apoptosis (Cheung et al. 2008; Lakner et al. 2011; Shah et al. 2013). The main activities of the most common miRNAs related to liver diseases are presented in Table 2 (Lakner et al. 2011). Nevertheless, the application of miRNAs for clinical therapy needs further investigation to better understand the significance in liver diseases and provide rational decision making for their utility as biomarkers.

Telomere Length

Telomeres are nucleoprotein structures that protect the ends of eukaryote chromosomes. Shorter telomere length is associated with some age-related human disorders, and their inverse association with changes in obesity parameters has been recently discovered. The assessment of telomere length can provide further insights for biological pathways leading to adiposity and disease risk (Garcia-Calzon et al. 2014). Recently, a nested case–control study among 140 hepatitis B virus-related HCC cases and 280 frequency-matched cancer-free controls evaluated the association between relative telomere length in circulating cell-free serum DNA and HCC risk. Interestingly, the study reported that longer relative telomere length conferred a significantly increased HCC risk compared to short telomere length. This association proved to be more pronounced in patients without cirrhosis compared with those with cirrhosis (Fu et al. 2012). Whether telomere length could potentially be influenced by obesity and metabolic dysfunction and its potential application as a novel noninvasive biomarker for non-cirrhotic HCC remains to be evaluated by future research.

Metabolic Biomarkers in Gallbladder Cancer

Despite the suggested link between obesity and gallbladder cancer risk (Schlesinger et al. 2013), there have been only a few studies aimed at identifying metabolic biomarkers in gallbladder cancer prediction and screening. The two studies investigating the association between blood metabolite levels included 184 and 368 cases, respectively. Both focused on metabolites related to the metabolic syndrome. The first study reported an association between triglyceride levels and gallbladder cancer risk, which could not be confirmed in a later study. The second study found an association between glucose levels and gallbladder cancer risk, but information on gallstones and other established gallbladder cancer risk factors was not available. To our knowledge only one report exists on the association between circulating miRNA levels and risk of gallbladder cancer as separated tumor entity. Only two studies have investigated the association between a small number of blood metabolite levels and gallbladder cancer risk. The first study identified eleven miRNAs, which were differentially expressed in whole blood of gallbladder cancer cases as compared to controls. This study relied on

Table 2 Selected miRNA activities in liver diseases (18). The table represents a list of selected miRNA activities in liver diseases as identified by the current literature. The respective miRNAs, the type of sample where these have been isolated, the description of activities, and role of dysregulation of the specific miRNAs in human diseases are summarized as per currently reported evidence. It can be observed that differentially expressed miRNAs in humans and animal models of NASH regulate genes with diverse functions involved in the pathogenesis of NAFLD, including metabolism of lipid and glucose, regulation of the unfolded protein response, endoplasmic reticulum stress, oxidative stress, cellular differentiation, inflammation, and apoptosis

miRNAs	Type of sample	Activities	Human diseases and dysregulation of miRNAs
MiR-122	Liver tissue	Regulation of total serum cholesterol and hepatic lipid metabolism, maintain the hepatic cell phenotype, influence on FA synthesis Correlates with necro-inflammatory activity. Regulate CYP7A1 bile acid synthesis. Also loss of miR-122 may lead to increased fibrosis through Klf6	Upregulated: steatosis and NASH, fibrosis, cirrhosis, HCC serum Downregulated: HCC tissue
MiR-34a	Liver tissue, blood	Regulation of total serum cholesterol and hepatic lipid metabolism, senescence, cell cycle arrest, and apoptosis by suppressing SIRT1	Upregulated: steatosis and NASH, fibrosis, cirrhosis Downregulated: HCC
MiR-16	Liver tissue, blood	Important in NFLD pathogenesis, correlating with liver inflammation	Upregulated: steatosis and NASH, HCC Downregulated: fibrosis
MiR-33a/b	Liver tissue	Promotion of cholesterol and FA synthesis, decreased plasma level of VLDL-associated triglycerides and increased HDL-associated triglycerides, bile acid regulation	Upregulated: steatosis and NASH, fibrosis, cirrhosis, HCC serum Downregulated: HCC
MiR-200	Liver tissue	Target various molecules involved in apoptosis, lipid and carbohydrate metabolism	Upregulated: steatosis and NASH, fibrosis Downregulated: HCC
MiR-99a/b	Liver tissue, adipose tissue	Inhibition of tumor growth by inducing cell cycle arrest, MiR-99a correlates negatively with FFA and IL-6, MiR-99b is associated with pericellular fibrosis in NASH	Downregulated: steatosis and NASH, fibrosis, HCC
MiR-21	Liver tissue, blood	Involvement in liver steatosis, first oncomir with target of tumor suppressor genes	Upregulated: fibrosis, cirrhosis, HCC Downregulated: steatosis and NASH
MiR-221	Liver tissue, serum	Involved in liver fibrosis	Upregulated: steatosis and NASH, fibrosis, cirrhosis, HCC

(*continued*)

Table 2 (continued)

miRNAs	Type of sample	Activities	Human diseases and dysregulation of miRNAs
MiR-155	Liver tissue	Role in the regulation of inflammation and tumorigenesis (upregulated at the early stage of NASH-induced hepatocarcinogenesis)	Upregulated: steatosis and NASH, fibrosis, cirrhosis, HCC
MiR-181a/b	Liver tissue	Associated with susceptibility to NAFLD and the extent of NAFLD associated liver injury, promote HSCs growth by targeting the cyclin-dependent kinase inhibitor 1B	Upregulated: steatosis and NASH, fibrosis, cirrhosis, HCC
MiR-10b	Liver tissue	New regulator of steatosis level, involved in postprandial regulation of the nuclear receptor peroxisome proliferator-activated receptor-α	Upregulated: steatosis and NASH, HCC
Let-7	Liver tissue	Play role during liver fibrosis and tumorigenesis by protecting human hepatocytes from oxidative stress and being suppressor of cell growth	Upregulated: steatosis and NASH, fibrosis, HCC Downregulated: steatosis and NASH, HCC
MiR-199a/b-3p	Liver tissue	Related to grade of liver fibrosis, independent predictor for reduced tumor-free survival in HCC individuals	Upregulated: steatosis and NASH, fibrosis Downregulated: HCC
MiR-128-2	Liver tissue	Control of cholesterol homeostasis might confer resistance to apoptosis and induce cancer, regulator of SIRT1	Downregulated: steatosis and NASH

40 gallbladder cancer cases and 40 healthy controls and provides promising evidence on blood miRNA expression levels as biomarkers for gallbladder cancer prevention. Further studies covering heterogeneous populations are warranted in order to identify novel biomarkers for gallbladder cancer. Biomarker discovery could offer huge potential for primary prevention and early diagnosis of this highly aggressive disease especially considering prophylactic cholecystectomy.

Potential Applications to Prognosis, Other Diseases, or Conditions

As discussed above, liver cancer is a complex disease caused by multiple risk factors which makes the early diagnosis and prognostic assessment difficult. New advancements on the differential etiological profile in Western populations characterized by

high obesity rates highlight the need to identify valuable prognostic biomarkers. A prognostic biomarker could be defined as a clinical or biologic characteristic that is objectively measurable and that provides information on the likely outcome of the cancer disease in an untreated individual. Such biomarkers are helpful for identifying patients with cancer who are at high risk of metastatic relapse and therefore potential candidates for systemic treatment. Circulating biomarkers such as serum alpha-fetoprotein are commonly used for diagnosis and surveillance of primary liver cancer, but also for prognosis as patients with a high serum alpha-fetoprotein levels were shown to have shorter survival. Metabolic biomarkers have been evaluated in a variety of other obesity-associated chronic diseases, including cardiovascular disease, diabetes, and cancer. However, whether biomarkers in obesity and metabolic dysfunction could serve as early prognostic makers for liver cancer survival is currently not clarified. Indeed, the overall influence of obesity in liver cancer survival remains controversial. A recent systematic review including a total of 14 studies suggested that BMI as an obesity indicator was not associated with survival (including overall and disease-free survival) and postoperative complications in liver cancer patients (Rong et al. 2015). Nevertheless, a recent cohort of 140 HCC patients provided first evidence on the association of metabolic biomarkers in HCC prognosis. The study reported that higher serum adiponectin level was independently associated with worsened overall survival even after adjusting for important clinical covariates. No association was revealed for other investigated metabolic biomarkers, including leptin and HOMA-IR (Siegel et al. 2015). The role of obesity and metabolic biomarkers in liver cancer progression and survival and their potential utility as prognostic factors remains to be elucidated.

Summary and Conclusion

Obesity, nonalcoholic fatty liver disease, and associated metabolic disorders are convincingly associated with in the risk of hepatobiliary tract cancers, particularly in “low-risk” Western populations such as Northern Europe and the United States. Recent evidence has also implicated biomarkers associated with obesity and impaired metabolism in the risk of hepatobiliary tract cancer development and progression. In particular, selected metabolic biomarkers could play a role as potential intermediates of the association between obesity and liver cancer. These include circulating biomarkers of hyperinsulinemia, insulin-like growth factors, C-peptide, and HOMA-IR index, and biomarkers of chronic low-grade inflammation and immune response, C-reactive protein and interleukin-6 and selected adipose tissue-derived cytokines and hormones. In addition, novel metabolites representing key metabolic alterations in amino acid, polyunsaturated lipid, acetate, and citrate metabolism have been recently identified as important predictors of liver cancer risk. Targeting metabolic abnormalities, such as attenuation of chronic inflammation and improvement of insulin resistance by either pharmaceutical or nutritional intervention, may be an effective strategy in preventing the development of HCC in obese individuals. Further research is warranted in order to better characterize specific

metabolic biomarkers in understanding etiology and their validation as early markers for risk assessment and prognosis of hepatobiliary tract cancer.

Summary Points

- Worldwide, primary liver cancer is the fifth most common cancer in men and the ninth most common cancer in women, representing the second leading cause of cancer-related death worldwide.
- Biomarkers for early risk assessment and diagnosis may provide means for improved prevention of this aggressive tumor.
- Recently the role of obesity and abdominal fat accumulation has been established in the development of liver cancer, particularly among "low-risk" populations such as Western countries (Northern Europe and the United States).
- Biomarkers in obesity-related metabolic pathways may assist in understanding the link between obesity and cancer as well as provide innovative alternatives for liver cancer prevention in Western populations.
- Current evidence has established biomarkers related to hyperinsulinemia, chronic low-grade inflammation and immune response, and selected adipose tissue-derived hormones to be associated with the risk of hepatocellular carcinoma.
- Metabolic alterations in amino acid, polyunsaturated lipid, acetate, and citrate metabolism have been recently implicated in the development of liver cancer risk.
- Metabolic biomarkers were suggested to improve liver cancer diagnosis beyond the most common HCC biomarkers in clinical practice – i.e., alpha-fetoprotein and liver enzyme levels.
- Drawing from current research, the present report suggests that targeting metabolic abnormalities, such as attenuation of chronic inflammation and improvement of insulin resistance by either pharmaceutical or nutritional intervention, may be an effective strategy in preventing the development of hepatobiliary cancer.
- The application of novel biomarkers identified using metabolomics, proteomics, and glycomics, as well as the suggested use of miRNAs for risk assessment and therapy, requires further investigation to better understand their clinical significance and methodological utility.

References

Abenavoli L, Peta V. Role of adipokines and cytokines in non-alcoholic fatty liver disease. Rev Recent Clin Trials. 2014;9(3):134–40.

Aleksandrova K, et al. Inflammatory and metabolic biomarkers and risk of liver and biliary tract cancer. Hepatology. 2014;60(3):858–71.

Arano T, et al. Serum level of adiponectin and the risk of liver cancer development in chronic hepatitis C patients. Int J Cancer. 2011;129(9):2226–35.

Bekaert M, et al. Association of recently described adipokines with liver histology in biopsy-proven non-alcoholic fatty liver disease: a systematic review. Obes Rev. 2016;17(1):68–80.

Bell LN, et al. Serum proteomics and biomarker discovery across the spectrum of nonalcoholic fatty liver disease. Hepatology. 2010;51(1):111–20.
Bellentani S, Marino M. Epidemiology and natural history of non-alcoholic fatty liver disease (NAFLD). Ann Hepatol. 2009;8 Suppl 1:S4–8.
Bertolani C, Marra F. The role of adipokines in liver fibrosis. Pathophysiology. 2008;15(2):91–101.
Buechler C, Wanninger J, Neumeier M. Adiponectin, a key adipokine in obesity related liver diseases. World J Gastroenterol. 2011;17(23):2801–11.
Cabibbo G, Craxi A. Epidemiology, risk factors and surveillance of hepatocellular carcinoma. Eur Rev Med Pharmacol Sci. 2010;14(4):352–5.
Caldwell SH, et al. Obesity and hepatocellular carcinoma. Gastroenterology. 2004;127(5 Suppl 1): S97–103.
Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer. 2004;4(8):579–91.
Center MM, Jemal A. International trends in liver cancer incidence rates. Cancer Epidemiol Biomarkers Prev. 2011;20(11):2362–8.
Chao LT, et al. Insulin, glucose and hepatocellular carcinoma risk in male hepatitis B carriers: results from 17-year follow-up of a population-based cohort. Carcinogenesis. 2011;32(6): 876–81.
Chen C, et al. Serum protein *N*-glycans profiling for the discovery of potential biomarkers for nonalcoholic steatohepatitis. J Proteome Res. 2009;8(2):463–70.
Chettouh H, et al. Hyperinsulinaemia and insulin signalling in the pathogenesis and the clinical course of hepatocellular carcinoma. Liver Int. 2015;35(10):2203–17.
Cheung O, et al. Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. Hepatology. 2008;48(6):1810–20.
Clemmons DR. Insulin-like growth factor binding proteins and their role in controlling IGF actions. Cytokine Growth Factor Rev. 1997;8(1):45–62.
Donohoe CL, Doyle SL, Reynolds JV. Visceral adiposity, insulin resistance and cancer risk. Diabetology & Metabolic Syndrome. 2011;3:12. doi:10.1186/1758-5996-3-12.
Dutta D, et al. Leptin and cancer: pathogenesis and modulation. Indian J Endocrinol Metab. 2012;16 Suppl 3:S596–600.
Eguchi Y, et al. Visceral fat accumulation and insulin resistance are important factors in nonalcoholic fatty liver disease. J Gastroenterol. 2006;41(5):462–9.
Eguchi Y, et al. The pathological role of visceral fat accumulation in steatosis, inflammation, and progression of nonalcoholic fatty liver disease. J Gastroenterol. 2011;46 Suppl 1:70–8.
Elinav E, et al. Suppression of hepatocellular carcinoma growth in mice via leptin, is associated with inhibition of tumor cell growth and natural killer cell activation. J Hepatol. 2006;44(3):529–36.
El-Serag HB. Hepatocellular carcinoma. N Engl J Med. 2011;365(12):1118–27.
El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology. 2007;132(7):2557–76.
Fages A, Duarte-Salles T, Stepien M, Ferrari P, Fedirko V, Pontoizeau C, et al. Metabolomic profiles of hepatocellular carcinoma in a European prospective cohort. BMC medicine. 2015;13:242. http://creativecommons.org/publicdomain/zero/1.0/.
Ferlay J et al. Cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet]. Lyon: International Agency for Research on Cancer; 2014. Available from http://globocan.iarc.fr. Accessed 30 May 2016.
Fitzpatrick E, Dhawan A. Noninvasive biomarkers in non-alcoholic fatty liver disease: current status and a glimpse of the future. World J Gastroenterol. 2014;20(31):10851–63.
Fu X, et al. Relative telomere length: a novel non-invasive biomarker for the risk of non-cirrhotic hepatocellular carcinoma in patients with chronic hepatitis B infection. Eur J Cancer. 2012; 48(7):1014–22.
Garcia-Calzon S, et al. Longitudinal association of telomere length and obesity indices in an intervention study with a Mediterranean diet: the PREDIMED-NAVARRA trial. Int J Obes (Lond). 2014;38(2):177–82.

GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 1 [Internet].Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on 25 May 2016.
Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. Am J Clin Nutr. 2006;83(2):461S–5.
Haukeland JW, et al. Fetuin A in nonalcoholic fatty liver disease: in vivo and in vitro studies. Eur J Endocrinol. 2012;166(3):503–10.
Huang Q, et al. Metabolic characterization of hepatocellular carcinoma using nontargeted tissue metabolomics. Cancer Res. 2013;73(16):4992–5002.
Hung TM, et al. Up-regulation of microRNA-190b plays a role for decreased IGF-1 that induces insulin resistance in human hepatocellular carcinoma. PLoS One. 2014;9(2):e89446.
Jemal A, et al. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69–90.
Jung UJ, Choi MS. Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. Int J Mol Sci. 2014;15(4):6184–223.
Kalafateli M, et al. Adipokines levels are associated with the severity of liver disease in patients with alcoholic cirrhosis. World J Gastroenterol. 2015;21(10):3020–9.
Kalhan SC, et al. Plasma metabolomic profile in nonalcoholic fatty liver disease. Metabolism. 2011;60(3):404–13.
Kamada Y, Takehara T, Hayashi N. Adipocytokines and liver disease. J Gastroenterol. 2008; 43(11):811–22.
Kamiyama T, et al. Identification of novel serum biomarkers of hepatocellular carcinoma using glycomic analysis. Hepatology. 2013;57(6):2314–25.
Lakner AM, Bonkovsky HL, Schrum LW. MicroRNAs: fad or future of liver disease. World J Gastroenterol. 2011;17(20):2536–42.
LeRoith D, et al. Insulin-like growth factors and cancer. Ann Intern Med. 1995;122(1):54–9.
Li H, et al. A proton nuclear magnetic resonance metabonomics approach for biomarker discovery in nonalcoholic fatty liver disease. J Proteome Res. 2011;10(6):2797–806.
Lim U, et al. Predicting total, abdominal, visceral and hepatic adiposity with circulating biomarkers in Caucasian and Japanese American women. PLoS One. 2012;7(8):e43502.
Marchesini G, et al. Obesity-associated liver disease. J Clin Endocrinol Metab. 2008;93(11 Suppl 1):S74–80.
Marra F, Tacke F. Roles for chemokines in liver disease. Gastroenterology. 2014;147(3):577–94.e1.
Michikawa T, et al. Plasma levels of adiponectin and primary liver cancer risk in middle-aged Japanese adults with hepatitis virus infection: a nested case–control study. Cancer Epidemiol Biomarkers Prev. 2013;22(12):2250–7.
Moschen AR, Wieser V, Tilg H. Adiponectin: key player in the adipose tissue-liver crosstalk. Curr Med Chem. 2012;19(32):5467–73.
Naim Alkhouri MD, Kay MH, FACG MD. The cleveland clinic, cleveland, OH – Updated Dec 2012. http://patients.gi.org/topics/fatty-liver-disease-nafld
Nkontchou G, et al. Insulin resistance, serum leptin, and adiponectin levels and outcomes of viral hepatitis C cirrhosis. J Hepatol. 2010;53(5):827–33.
Ohishi W, et al. Serum interleukin-6 associated with hepatocellular carcinoma risk: a nested case–control study. Int J Cancer. 2014;134(1):154–63.
Park JE, et al. Differential expression of intermediate filaments in the process of developing hepatic steatosis. Proteomics. 2011;11(14):2777–89.
Polyzos SA, et al. The potential adverse role of leptin resistance in nonalcoholic fatty liver disease: a hypothesis based on critical review of the literature. J Clin Gastroenterol. 2011;45(1):50–4.
Polyzos SA, Kountouras J, Mantzoros CS. Adipokines in nonalcoholic fatty liver disease. Metabolism. 2015;56:8029.
Polyzos SA, et al. Circulating leptin in non-alcoholic fatty liver disease: a systematic review and meta-analysis. Diabetologia. 2016;59(1):30–43.

Qiao L, Li X. Role of chronic inflammation in cancers of the gastrointestinal system and the liver: where we are now. Cancer Lett. 2014;345(2):150–2.
Ribatti D, et al. Leptin-leptin receptor are involved in angiogenesis in human hepatocellular carcinoma. Peptides. 2008;29(9):1596–602.
Roessner U, et al. Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. Plant Cell. 2001;13(1):11–29.
Rong X, et al. The association between body mass index and the prognosis and postoperative complications of hepatocellular carcinoma: a meta-analysis. Medicine (Baltimore). 2015; 94(31):e1269.
Sayed D, Abdellatif M. MicroRNAs in development and disease. Physiol Rev. 2011;91(3):827–87.
Scharf JG, Ramadori G, Dombrowski F. Analysis of the IGF axis in preneoplastic hepatic foci and hepatocellular neoplasms developing after low-number pancreatic islet transplantation into the livers of streptozotocin diabetic rats. Lab Invest. 2000;80(9):1399–411.
Schlesinger S, et al. Abdominal obesity, weight gain during adulthood and risk of liver and biliary tract cancer in a European cohort. Int J Cancer. 2013;132(3):645–57.
Shah NR, Braverman ER. Measuring adiposity in patients: the utility of body mass index (BMI), percent body fat, and leptin. PLoS One. 2012;7(4):e33308.
Shah N, Nelson JE, Kowdley KV. MicroRNAs in liver disease: bench to bedside. J Clin Exp Hepatol. 2013;3(3):231–42.
Shen J, et al. Non-invasive diagnosis of non-alcoholic steatohepatitis by combined serum biomarkers. J Hepatol. 2012;56(6):1363–70.
Siegel AB, et al. Serum adiponectin is associated with worsened overall survival in a prospective cohort of hepatocellular carcinoma patients. Oncology. 2015;88(1):57–68.
Stauffer JK, et al. Chronic inflammation, immune escape, and oncogenesis in the liver: a unique neighborhood for novel intersections. Hepatology. 2012;56(4):1567–74.
Stepien M, et al. Alteration of amino acid and biogenic amine metabolism in hepatobiliary cancers: findings from a prospective cohort study. Int J Cancer. 2016;138(2):348–60.
Stojsavljevic S, et al. Adipokines and proinflammatory cytokines, the key mediators in the pathogenesis of nonalcoholic fatty liver disease. World J Gastroenterol. 2014; 20(48):18070–91.
Torre LA, et al. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65(2):87–108.
van Dijk SJ, et al. Plasma protein profiling reveals protein clusters related to BMI and insulin levels in middle-aged overweight subjects. PLoS One. 2010;5(12):e14422.
Vanni E, et al. From the metabolic syndrome to NAFLD or vice versa? Dig Liver Dis. 2010; 42(5):320–30.
Vincent R, Sanyal A. Recent advances in understanding of NASH: microRNAs as both biochemical markers and players. Curr Pathobiol Rep. 2014;2(3):109–15.
von Loeffelholz C, et al. Fetuin A is a predictor of liver fat in preoperative patients with nonalcoholic fatty liver disease. J Invest Surg. 2016;16:1–9.
Wieser V, Moschen AR, Tilg H. Adipocytokines and hepatocellular carcinoma. Dig Dis. 2012; 30(5):508–13.
World Cancer Research Fund International/American Institute for Cancer Research. Continuous update project report: diet, nutrition, physical activity and liver cancer. 2015. Available at www.wcrf.org/sites/default/files/Liver-Cancer-2015-Report.pdf. Accessed 30 April 2016.
World Health Organization February 2015. Fact sheet: cancer. Available online http://www.who.int/mediacentre/factsheets/fs297/en/. Accessed 30 Aug 2015.
Wree A, et al. Obesity affects the liver – the link between adipocytes and hepatocytes. Digestion. 2011;83(1–2):124–33.
Yu C, et al. Serum proteomic analysis revealed diagnostic value of hemoglobin for nonalcoholic fatty liver disease. J Hepatol. 2012;56(1):241–7.

Biomarkers of the Antioxidant Response: A Focus on Liver Carcinogenesis

38

Ricardo Sánchez-Rodríguez, Julia Esperanza Torres-Mena, Luis del Pozo Yauner, and Julio Isael Pérez-Carreón

Contents

R. Sánchez-Rodríguez (✉) • J.E. Torres-Mena • L. del Pozo Yauner • J.I. Pérez-Carreón (✉)
Laboratorio de Bioquímica y Estructura de Proteínas, Instituto Nacional de Medicina Genómica, Mexico City, Mexico
e-mail: richikrdo@comunidad.unam.mx; jiperez@inmegen.gob.mx

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_36

Abstract

Many studies have demonstrated the association of oxidative stress caused by excessive and sustained production of reactive species with chronic inflammatory conditions, neurodegenerative diseases, diabetes mellitus, atherosclerosis, and cancer. The main mechanism of redox control relies on the cellular antioxidant response. Nevertheless, oxidative stress and oxidative damage to biomolecules are events inherent to the process of carcinogenesis. Antioxidant response systems do not operate in isolation, as there is significant convergence among thermodynamically favored systems. Three main systems may be identified: glutathione, thioredoxins (TRX), and nicotinamide adenine dinucleotide phosphate (NADPH). Liver tumors frequently exhibit overexpression of one or more proteins belonging to the antioxidant system, for example, glutathione reductase (GSR), glutathione S-transferase P (GSTP), gamma-glutamyl transferase (GGT), glucose-6-phosphate dehydrogenase (G6PD), thioredoxin reductase (TXNR), NAD(P)H dehydrogenase [quinone] 1 (NQO1), and prostaglandin reductase 1 (PTGR1). The increased expression of these enzymes is suggested as biomarker that favors tumor development by stimulating proliferation, angiogenesis, and metastasis or by preventing cell death. The loss of expression of some antioxidant enzymes could be used as biomarkers too such as catalase (CAT) and superoxide dismutase (SOD). Therefore, the expression of these proteins shows predictive value for the prognosis and risk of liver cancer recurrence in patients. In this chapter, we discuss the most recent findings regarding the enzymatic antioxidant cellular response that occurs during liver carcinogenesis and how these systems could be used as biomarkers in the clinical practice.

Keywords

Liver cancer • Oxidative stress • Antioxidant response • Redox state • Reactive oxygen and nitrogen species

List of Abbreviation

4-HNE	4-Hydroxynonenal
8-OH-dG	8-hydroxy-20-deoxyguanosine
AKR	Aldo-keto reductase
ATF-2	Activating transcription factor

ATM	Ataxia telangiectasia mutated
CAT	Catalase
CBR1	Carbonyl reductase 1
G6PD	Glucose-6-phosphate dehydrogenase
GCL	Glutamate cysteine ligase
GGT	Gamma-glutamyl transferase
GPX	Glutathione peroxidase
GS	Glutathione synthetase
GSH and GSSG	Reduced and oxidized glutathione
GSR	Glutathione reductase
GST	Glutathione S-transferases
HCC	Hepatocellular carcinoma
HIF	Hypoxia-inducible factors
HMOX	Heme oxygenase
HNF4α	Hepatocyte nuclear factor alpha
MDA	Malondialdehyde
MDR	Multidrug resistance protein
MT	Metallothionein
NADPH	Nicotinamide adenine dinucleotide phosphate
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2
NF-kB	Nuclear factor kB
NQO1	NADPH quinone oxidoreductase 1
PI3K	Phosphoinositide 3 kinase
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptors
PRX	Peroxiredoxin
PTEN	Phosphatase and tensin homolog
PTGR	Prostaglandin reductase
RAR	Retinoic acid receptors
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TRX	Thioredoxin
TXNR	Thioredoxin reductase

Key Facts of the Antioxidant Response

- Cells possess a system for the direct elimination of reactive species that is called antioxidant response.
- Antioxidant response is an important part of the cell's defense against oxidative stress.

- Antioxidant response is composed of transcription factors, enzymes, proteins, and peptides that protect against oxidative stress.
- Oxidative stress is closely associated with the development of cancer, but tumoral cells overexpress several proteins of the antioxidant response for their own protection.
- Levels of reactive species and antioxidant response determine the cellular oxide-reduction state.

Definition of Words and Terms

4-hydroxynonenal (4-HNE)	Lipid peroxidation product, which may serve as markers of oxidative stress and it can induce cell death.
Antioxidant response	Cellular system for the direct elimination of reactive species.
Glutathione	Tripeptide of glutamate, glycine, and cysteine, which include a non-proteic thiol group, glutathione plays a fundamental role in the maintenance of a reduced cellular state.
Metallothionein	Low-molecular-weight proteins that are expressed in response to stress. These proteins exhibit conserved cysteine-rich domains that are able to bind to metals to play a role in metal homeostasis.
NADPH	Nicotinamide adenine dinucleotide phosphate. It is tightly associated with the glutathione and thioredoxin systems in the maintenance of the oxidation-reduction (REDOX) balance. Additionally, NADPH is used as a cofactor by a variety of enzymes that mediate oxidation-reduction reactions as part of the phase II antioxidant response.
Oxidative stress	The loss of oxidation-reduction balance caused by either an increase in oxidant species or deficiencies in cellular antioxidant molecules.
Reactive species	Free radicals and their metabolites that are able to chemically modify various biomolecules, such as lipids, proteins, and DNA.
Redox state	Oxide and reduction status in the cells.
Thioredoxins	A group of proteins involved in oxidation-reduction recycling dependent on NADPH and these proteins exhibit in common a conserved cysteine domain.

Introduction

Reactive Oxygen and Nitrogen Species

Reactive species, or free radical molecules, are able to chemically modify various biomolecules, such as lipids, proteins, and DNA (Valko et al. 2007). A free radical is a highly reactive species with atomic orbits containing one or more unpaired electrons that are able to exist independently (Halliwell and Gutteridge 1984). Generally, free radicals and their metabolites are considered reactive species. Free radicals may be generated naturally within biological systems. The most common reactive species are those derived from oxygen (reactive oxygen species, ROS) and those derived from nitrogen (reactive nitrogen species, RNS), although reactive species derived from iron and copper also exist (Valko et al. 2007).

ROS include superoxide anions, hydrogen peroxide, hydroxyl radicals, singlet oxygen, and organic peroxides. ROS may be endogenously generated by the cell, via the activity of enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cytochrome P450, xanthine oxidase, or myeloperoxidase or through the activity of the mitochondrial electron transport chain. ROS may also be generated exogenously, for example, by ionizing radiation (Oyagbemi et al. 2009). The main representative species of RNS is nitric oxide, the product of the activity of nitric oxide synthase (NOS). Additionally, nitric oxide can react with superoxide to generate peroxy nitrites (Valko et al. 2007).

Reactive species may generate different metabolites upon reacting with biomolecules. For example, the reaction between reactive species and cell membrane polyunsaturated fatty acids can produce reactive metabolites with a mutagenic capacity, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which may serve as markers of oxidative stress (Wang and Liehr 1995; Bartsch et al. 2002). Oxidized nucleotides such as mutagenic 8-hydroxy-20-deoxyguanosine (8-OH-dG) are formed by the reaction of reactive species with DNA (Droge 2002; Murtas et al. 2010). Finally, 3-nitrotyrosine is formed by the reaction of RNS with proteins (Sainz et al. 2012).

Oxidative Stress

Oxidative stress is defined as the loss of oxidation-reduction balance, caused by either an increase in oxidants or deficiencies in cellular antioxidants, leading to increased levels of reactive species (Droge 2002; Valko et al. 2007; Sainz et al. 2012). Therefore, oxidative stress results from an imbalance in the redox state favoring an oxidative cellular environment.

The relationship between reduced (GSH) and oxidized (GSSG) glutathione is regarded as the best parameter for measuring the presence of cellular oxidative stress in an organism. Other such parameters include depletion of GSH (Valko et al. 2007; Franco and Cidlowski 2012) and elevated levels of MDA, 4-HNE, 8-OH-dG, and 3-nitrotyrosine.

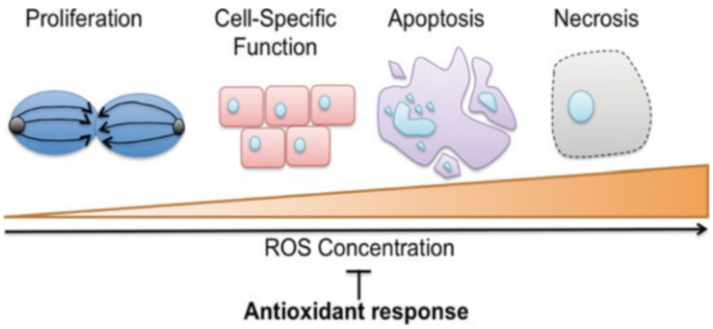

Fig. 1 ROS concentration modulates cellular functions from cell proliferation to cell death. Cell proliferation, the cell-specific function, apoptosis, and necrosis are regulated through cellular signaling, where functions of proteins and metabolites can be controlled by the oxidized and reduced state. Thus the ROS stimulus plays an important role, which can be modified by the antioxidant response

Cell Signaling and Redox Status

Several studies have demonstrated that the regulation of the redox state is important for the modulation of cellular functions, in both normal and tumor cells. Cellular signaling is altered or modified according to the concentration and duration of signals produced directly by reactive species or via the reduction of antioxidants such as GSH (Fig. 1). For example, ROS and RNS have the capacity to stimulate the release of pro-inflammatory cytokines such as IL-6 or TNF-α and anti-inflammatory cytokines such as IL-10, resulting in modulation of the immune response. Moreover, ROS may contribute to the processes of angiogenesis and cell death in a concentration-dependent fashion. For example, 4-HNE can activate pathways leading to cell rescue, or it may induce apoptosis, necrosis, senescence, or autophagy (Valko et al. 2007; Dalleau et al. 2013). Reactive species can directly oxidize protein residues, inducing changes in protein activity, as occurs in the EGF, insulin, PKC, and MAPK (Droge 2002) signaling pathways.

Proteins Regulated by the Redox State

Reactive species may induce other types of posttranslational modifications. For example, the S-nitrosylation (Sengupta and Holmgren 2012) or S-glutathionylation (Pallardo et al. 2009) of proteins (the latter of which is mediated by GSH) is dependent on the oxidized/reduced state of cysteine residues. The oxidation of cysteine residues can generate sulfenic, sulfinic, and sulfonic groups, based on the degree of oxidation, and influences the redox potential of the protein. As described below, these modifications play important roles in protein signaling and inactivation. Additionally, glutathionylation of DNA and histones is thought to be relevant to the epigenetic regulation of gene expression (Pallardo et al. 2009; Zhang and Forman 2012). The proteins ATM, PLC, PI3K, PTEN, and HIF are sensitive to the

concentration of GSH, which is generally associated with hypoxia due to the failure of respiratory chain complex III (Bae et al. 2011). The concentration of GSH is closely correlated with the level of reactive species and contributes to various cellular processes. For example, low levels of GSH favor the activation of apoptosis or cell cycle arrest (Reddy et al. 2008; Franco and Cidlowski 2012). In contrast, increased GSH levels favor resistance against apoptosis. The nucleus/cytoplasm ratio of GSH levels is essential for cell cycle regulation (Pallardo et al. 2009); as this ratio is influenced by fluctuations in the redox environment, it affects functional role of cell cycle proteins such as cdks, p53, and p21 (Valko et al. 2007).

Antioxidant Response

Cells possess a system for the direct elimination of reactive species. This system includes various antioxidant enzymes, such as superoxide dismutase, catalase, and peroxidases, as well as other proteins with oxidoreductase activity or with thioredoxin domains. Moreover, nonenzymatic systems such as the GSH system allow the redox homeostasis within cells to be maintained. The expression of these proteins is regulated by the activation of transcription factors such as NFE2L2, NF-kB, and AP-1, which modulate the presence of reactive species. A global view of the antioxidant response system is illustrated in Figs. 2 and 3. Antioxidant response systems do not operate in isolation, as there is significant convergence among thermodynamically favored systems. Three main systems may be identified: glutathione, thioredoxins, and NADPH (Penney and Roy 2013).

Stress and Oxidative Damage in Carcinogenesis

Studies on carcinogenesis, particularly those involving chemical compounds, have demonstrated a correlation between persistent oxidative stress and damage to DNA, proliferation, adhesion, and cell survival. Moreover, persistent oxidative stress is implicated in the inactivation of tumor suppressor genes, overexpression of proto-oncogenes, and genetic instability. Sustained DNA damage alters signal transduction and transcription, increases the frequency of errors during replication, and induces genetic instability, ultimately promoting carcinogenesis. High levels of ROS are essential during the initiation and promotion stages of chemical carcinogenesis. Nevertheless, recent findings support the role of antioxidants in the carcinogenesis process (Saeidnia and Abdollahi 2013; Sayin et al. 2014), possibly via perturbation of the redox equilibrium or the production of stable radical species. Therefore, maintenance of the redox equilibrium is essential for cellular adaptation and survival. Perturbation of the redox equilibrium may lead to the activation of specific signaling pathways, depending on the signal type, duration, and intensity affecting the redox balance. Thus, numerous components of oxidative stress such as 4-HNE and antioxidant pathways such as the glutathione metabolism can be used as biomarkers of tissue damage or disease state (Ooi et al. 2011).

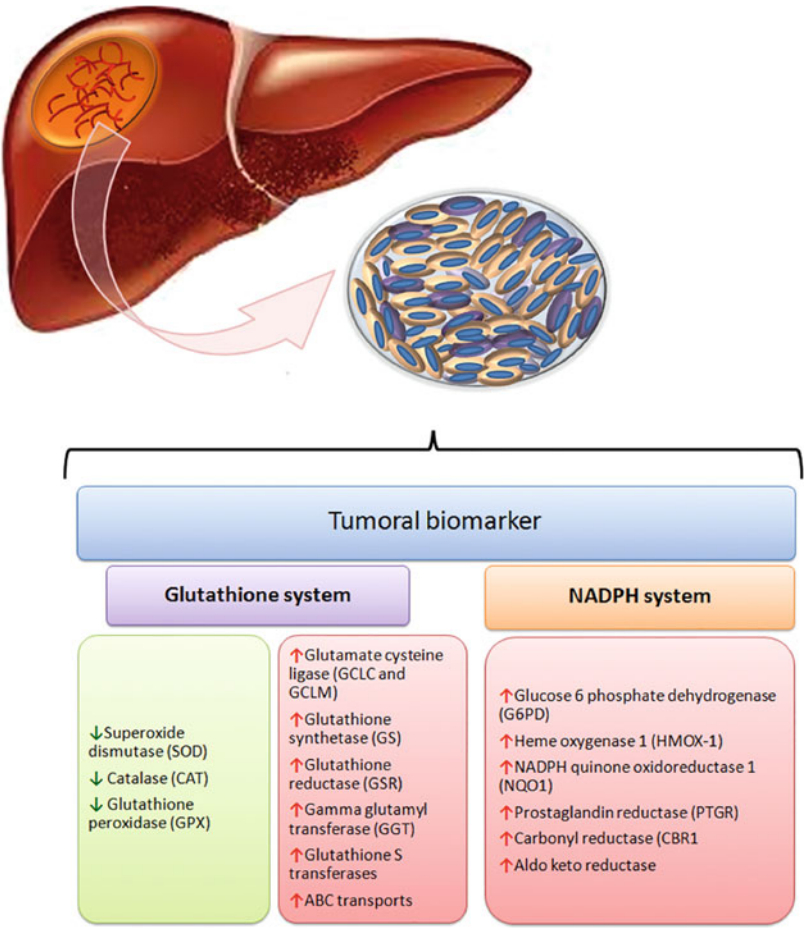

Fig. 2 Proteins of the antioxidant response could be tumoral biomarkers. The antioxidant proteins can modulate the oxidation-reduction (REDOX) cellular equilibrium. The loss or reduced expression of these enzymes is generally associated with an increased risk of cancer development; however their overexpression in tumors has been associated with poor prognosis, resistance to chemotherapy, and recurrence. Thus, they could be good biomarkers of cellular perturbations

Oxidative Stress and Liver Cancer

Hepatocellular carcinomas (HCC) and cholangiocarcinomas are the most frequent types of liver carcinomas, and their classification is dependent on the type of cells that give rise to these tumors (Llovet et al. 2003). Risk factors for liver cancer include hepatitis B (HBV) and hepatitis C (HCV) viral infections, alcoholic cirrhosis, metabolic syndrome, biliary cirrhosis, chronic hepatic lesions, hemochromatosis, and consumption of foods contaminated with aflatoxin B1 (El-Serag and

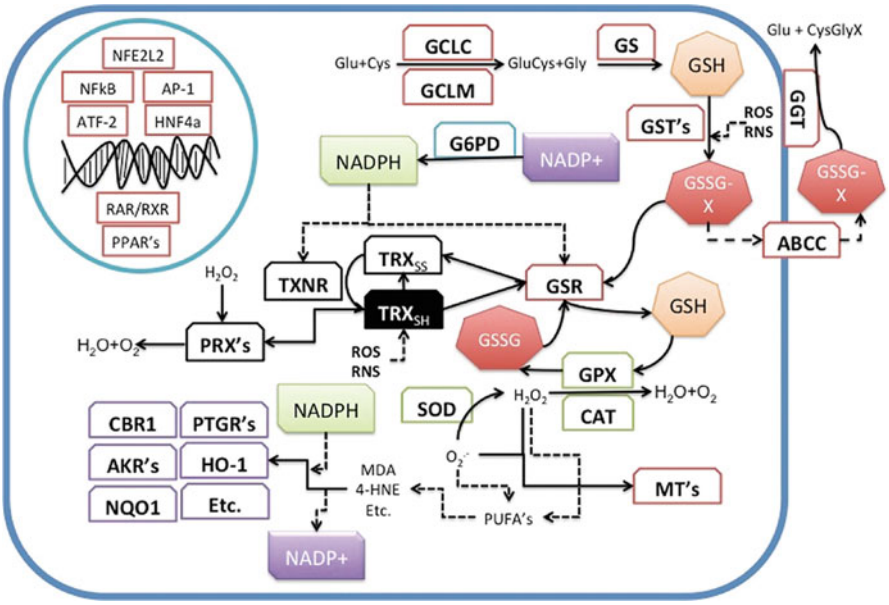

Fig. 3 Principal pathways of the antioxidant response. The transcription factors NFE2L2, NF-kB, AP-1, ATF-2, HNF4a, RAR/RXR, and PPARs are the main factors that regulate the expression of the proteins constituting the cellular antioxidant system. Various pathways for the detoxification of reactive species are interconnected with the GSH, NADPH, and thioredoxin (TRX) systems. The concentrations of ROS, O_2^{-}, and H_2O_2 can be directly modulated by enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), peroxiredoxin (PRX), and metallothioneins (MTs). The concentration of the remaining ROS and RNS may be regulated via conjugation with GSH (GS-X) mediated by GSTs or reduced by TRX, or these species may be metabolized by enzymes with oxidoreductase activity. A sustained antioxidant response may trigger an increase in the synthesis of GSH or TRX or may lead to activation of recycling pathways mediated by thioredoxin reductase (TXNR) and glutathione reductase (GSR) enzymes, which depend on the presence of NADPH. Alternatively, increased GSH synthesis may result from the recycling of amino acids such as glutamate via the activity of gamma-glutamyl transferase (GGT)

Rudolph 2007). These risk factors are diverse, but they share a similar tumorigenic mechanism that is dependent on the generation of oxidative stress. This has been demonstrated in experimental models of chemical hepatocarcinogenesis, in which ROS (Sanchez-Perez et al. 2005) are formed through the metabolic activation of chemical carcinogens, leading to the oxidation of lipids and proteins.

Antioxidant Response Enzymes as Biomarkers

Antioxidant Enzymes

The main enzymes involved in the antioxidant response are superoxide dismutase, catalase, and glutathione peroxidase (GPX). These enzymes are able to directly metabolize ROS, modulating the oxidation-reduction balance in cells, and loss or

Table 1 Antioxidant response enzymes used as a biomarker

Antioxidant enzyme	Gain or loss	Type of biomarker	References
SOD	Loss	Increase oxidative stress	(Marra et al. 2011)
SOD	Gain	Reduces tumor aggressiveness, good response to treatment, and prolonging survival time in patients	(Fu et al. 2011)
CAT	Loss	Increase oxidative stress	(Matos et al. 2009)
CAT	Gain	Positive response to treatment	(Matos et al. 2009)
GPX	Loss	Tumor progression	(Czeczot et al. 2006)

reduced expression of these enzymes is generally associated with an increased risk of cancer development. They are thus good biological indicators of cellular perturbations, in other words biomarkers (Fig. 2 and Table 1).

Superoxide Dismutase

SOD represents the main mechanism for the elimination of superoxide radicals in the mitochondria, through the activity of the xanthine oxidase and NADPH oxidase system, whereby superoxide radicals are transformed into hydrogen peroxide. Cells contain two main superoxide dismutase enzymes: SOD Cu/Mg or SOD1, localized to the cytoplasm, and SOD Mn or SOD2, localized to the mitochondria (Droge 2002). In general, an absence of SOD or reduced SOD activity is a marker that is associated with increased oxidative stress. In contrast, SOD expression reduces tumor aggressiveness, increasing treatment effectiveness and prolonging survival time in patients.

Catalase

The CAT protein participates in the metabolism of hydrogen peroxide, generated by peroxisomes via the activity of SOD or the cytochrome P450 system. Metabolism of hydrogen peroxide by CAT results in the production of water and oxygen. In hepatocellular carcinoma, reduced levels of CAT have been found within tumors or marker of increased levels of reactive species in neoplastic cells. CAT re-expression has been indicated as a possible biomarker for a positive response to treatment (Matos et al. 2009).

Glutathione Peroxidase

The enzyme GPX belongs to a family of enzymes containing eight isoforms that specialize in the catabolism of hydrogen peroxide using the tripeptide glutathione (GSH) as an electron donor (Valko et al. 2007; Reszka 2012). The expression of GPX is heterogeneous in solid tumors. Although the activity of GPX increases

Table 2 Phase II detoxification enzymes used as biomarker

Enzyme	Gain or loss	Type of biomarker	References
GCLC-GCLM	Gain	Biomarker of HCC	(Cheng et al. 2015)
GSR	Gain	Biomarker of tumor tissue	(Kekec et al. 2009)
GGT	Gain	Biomarker of tumorigenesis	(Corti et al. 2010; Zhang and Forman 2012)
ABCC3	Gain	Liver damage, resistance to drug treatment in HCC	(Colak et al. 2010; Zuniga-Garcia et al. 2015)
PRDX1	Gain	Angiogenesis in cancer	(Sun et al. 2014)
TRX1	Gain	Poor prognosis in secondary tumors	(Noike et al. 2008)
HMOX-1	Gain	Resistance to treatment and increased invasiveness in HCC	(Cheng et al. 2015)
NQO1	Gain	Resistance to treatment and poor prognosis in liver tumors	(Petrelli et al. 2014) (Wakai et al. 2011; Buranrat et al. 2012)
PTGR1	Gain	Biomarker of HCC	(Sanchez-Rodriguez et al. 2014)
CBR1	Gain	Progression of HCC	(Tak et al. 2011)
AKR1B10	Gain	Biomarker and progression of HCC	(Heringlake et al. 2010)

globally, specific isoforms may show reduced expression mediated by mechanisms of DNA hypermethylation.

In liver cancer, it has been found that global GPX activity as a biomarker is reduced compared with healthy and cirrhotic tissue (Czeczot et al. 2006). Nevertheless, the expression of GPX2 increases in liver tumors from the initial stages of hepatocarcinogenesis up to the final stages of metastases (Suzuki et al. 2013).

Phase II Detoxification Enzymes in Antioxidant Systems

The phase II detoxification enzymes are considered antioxidant enzymes due to their capacity to metabolize and eliminate reactive species, maintaining the cellular redox equilibrium. The majority of these enzymes participate in complexes converging on thioredoxins and nonprotein molecules such as GSH and NADPH, and they could be used as biomarker (Fig. 2 and Table 2).

Biomarkers of the Intracellular Glutathione System

Glutathione in its reduced form (GSH), with a non-proteic thiol group, is the tripeptide that is most abundant in cells (Franco and Cidlowski 2012). GSH is the main reducing agent and antioxidant involved in the fine control of the cellular redox status and plays a fundamental role in the maintenance of a reduced cellular state.

Changes in the intracellular balance of GSH/GSSG are the best biomarkers that determine the cellular redox status (Droge 2002; Pallardo et al. 2009; Lin et al. 2014); in fact, cytosolic depletion of GSH is the main characteristic of cell death by apoptosis (Circu and Aw 2008).

GSH depletion caused by oxidative stress or via active flow through the cell membrane generates an imbalance between reduced/oxidized glutathione (GSH/GSSG), resulting in the formation of reactive nitrogen species. GSH is able to induce posttranslational modifications in proteins via glutathionylation, a process that is dependent on the reduced/oxidized status of cysteine residues and the redox potential of the protein (Reddy et al. 2008; Franco and Cidlowski 2012). Therefore, the pathways for the synthesis and recycling of GSH play a central role in the antioxidant response. In solid tumors, GSH and dependent pathways are active in the regulation of the redox status, and abundant GSH confers greater resistance to antitumor treatment and reactive species; moreover, it results in greater tumor aggressiveness. Therefore, we next focus on the antioxidant enzymes that participate in the GSH system, reported to be associated with liver cancer (Fig. 4).

Glutamate Cysteine Ligase

The glutamate cysteine ligase (GCL) complex catalyzes the first and rate-limiting step in the synthesis of GSH: the union of the glutamate and cysteine at the gamma position. Glutamate cysteine ligase consists of a heterodimer with a catalytic subunit (GCLC) and a modifier subunit (GCLM) (Mougiakakos et al. 2012).

During the development of HCC, the expression of the GCLC and GCLM subunits increases from the early stages of preneoplastic lesions, and high expression levels are maintained during carcinogenesis in experimental models (Perez-Carreon et al. 2006; Albrethsen et al. 2011). In clinical samples of HCC, the GCLC and GCLM subunits were found to be under- and overexpressed in tumors, respectively, compared with cirrhotic tissue (Cheng et al. 2015) suggesting their use as biomarker for HCC. In contrast, in glioblastomas, inhibition of GCLC-GCLM complexes has been observed and is thought to be induced by posttranslational modifications induced by aldehydes (Backos et al. 2013). Therefore, another aspect that is important for understanding the functionality of enzymes involved in the antioxidant response in liver cancer is their transcriptional and transductional regulation.

Glutathione Synthetase

Glutathione synthetase (GS) catalyzes the second step in the synthesis of GSH, forming the peptide bond between γ-glutamyl-cysteine and glycine, resulting in the tripeptide (Franco and Cidlowski 2012). In experimental models of hepatocellular carcinoma, an increase in GS is observed from preneoplastic stages to the cancer

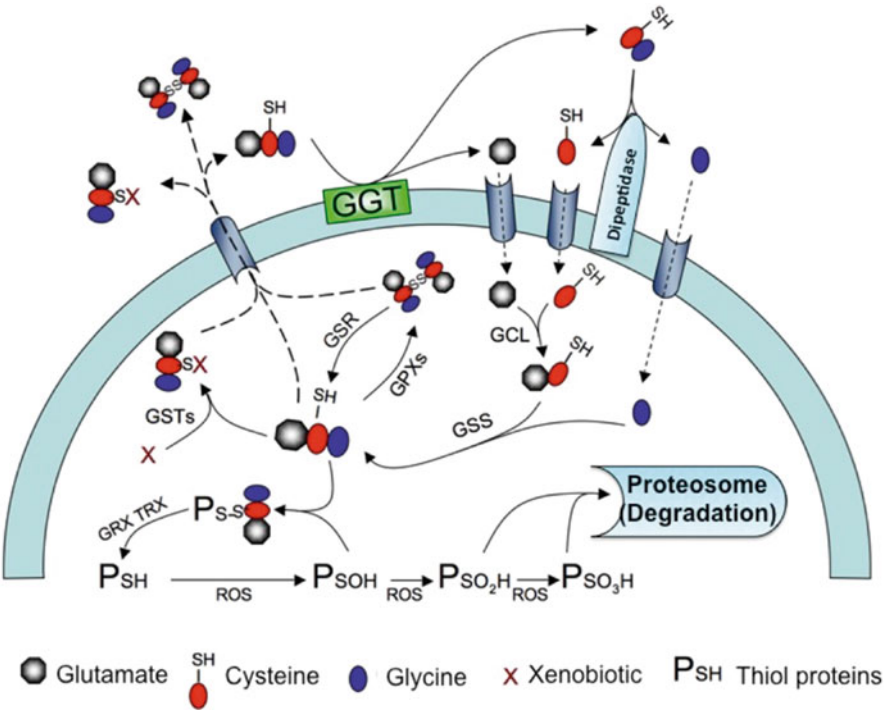

Fig. 4 Glutathione metabolism. GGT catabolizes extracellular glutathione via transfer of the gamma-glutamic group, and dipeptidases hydrolyze the cysteinylglycine. The synthesis of glutathione occurs intracellularly in two steps and catalyzed by the enzymes γ-glutamate cysteine ligase (GCL) and glutathione synthetase (GS). Under conditions of oxidative stress, GSH may be oxidized into GSSG via the activity of glutathione peroxidase (GPX) and reduced via the activity of GSR. One function of GSH is the detoxification of xenobiotics (X) through conjugation reactions, forming GS-X, which is performed by glutathione S-transferase enzymes (GSTs). Glutathione conjugates (GSH and GSSG) may be exported from the cell via glutathione transporters. Reactive oxygen species (ROS) may oxidize protein thiol groups (P-SH) to form proteins with sulfenic (P-SOH), sulfinic (P-SO_2H), and sulfonic (P-SO_3H) acid groups

stage. GS overexpression has also been observed during hepatic regeneration, which is differentiated only by a specific splice variant (Uchida et al. 2010). Therefore, future studies should investigate the types of GS isoforms present in liver tumors to be used as markers.

Glutathione Reductase

Glutathione reductase (GSR) belongs to the GSH recycling system and is involved in catalyzing the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as the electron donor. In gastric cancer, hepatocellular

carcinoma, and lung cancer, an increase in the activity of GSR is a biomarker in tumor tissue (Kekec et al. 2009; Sorokina et al. 2010; Pastor et al. 2013).

Gamma-Glutamyl Transferase

GGT is a membrane protein that catalyzes the degradation of extracellular glutathione and conjugates with glutathione between glutamate and cysteine residues. The activity of GGT favors the recovery of free amino acids, which may be used in subsequent intracellular GSH resynthesis (Corti et al. 2010). Work conducted by Hanigan and collaborators several decades ago demonstrated the presence of GGT in various types of solid tumors, such as renal carcinoma, papillary thyroid carcinoma, lung adenocarcinoma, hepatocellular carcinoma, and pancreatic, colon, prostate, ovary, breast, skin, and brain cancer (Hanigan et al. 1994; Kim et al. 2012). In scientific research, GGT is considered a biomarker of tumorigenesis (Corti et al. 2010; Zhang and Forman 2012).

Glutathione S-Transferases

Glutathione S-transferases (GST) are a family of enzymes that catalyze the conjugation of GSH to a variety of endogenous and exogenous molecules. The activity of these enzymes favors the elimination of xenobiotics (X). Glutathione S-transferases may be divided into five classes (α, μ, π, σ, and τ) (Valko et al. 2007). In both clinical and experimental HCC, reduced global activity of GST has been observed within tumors (Czeczot et al. 2006), and the specific isoforms GSTπ1 and GSTα1 (Colak et al. 2010; Albrethsen et al. 2011; Suzuki et al. 2013) have been shown to be overexpressed and have been used as biomarkers in experimental models of HCC.

ABC Transporters

ABC transporters are the best-studied proteins in regard to the development of resistance to antitumor pharmacological treatments and may also be referred to as multidrug resistance proteins (MDRs). This group is encoded by 48 genes, divided among seven families, and is involved in the transport of GSH and GSH conjugates (Chen and Tiwari 2011; Ooi et al. 2011); therefore, these proteins contribute to the regulation of the antioxidant response by eliminating toxic products and controlling the GSH/GSSG balance. As indicated by their name (MDRs), the expressions of these proteins in solid tumors are the biomarkers for resistance to drug treatment and greater rates of recurrence. In liver cancer, overexpression of ABCC9 has been observed. Overexpression of ABCC3 has been detected in both experimental and clinical HCC. ABCC3 has also been found to be overexpressed in

cholangiocarcinoma as well as in cirrhotic tissue and tissue infected with HVB (Colak et al. 2010; Yu et al. 2012; Zuniga-Garcia et al. 2015) suggesting as biomarker for damage to liver tissue.

Thioredoxin Domain Proteins as Biomarkers

This antioxidant response system consists of a group of proteins such as TRX and peroxiredoxins (PRXs) as well as proteins involved in oxidation-reduction recycling dependent on NADPH, as is the case for TXNR. These proteins exhibit in common a conserved cysteine domain that is capable of modulating reactive species and regulating the cellular redox status, and this domain allows these proteins to participate in various cellular processes, such as apoptosis and DNA synthesis. The lethality of TRX knockout in mice (Sengupta and Holmgren 2012; Penney and Roy 2013) demonstrated the importance of this protein for biological systems. Overexpression of one or more TRX proteins in cancer is common, suggesting that this system may play an important role in metabolism within tumor tissue. An important challenge in research is functional analysis of the activities of the various TRX isoforms and elucidating the specific functional differences between these isoforms.

In hepatocellular carcinoma, PRDX1 is overexpressed in tumors and is a biomarker associated with angiogenesis and cancer progression (Sun et al. 2014). In contrast, in secondary tumors resulting from colon cancer metastases, overexpression of TRX1 is common and is a biomarker associated with a poor prognosis (Noike et al. 2008). Overexpression of TRX has been observed in preneoplastic lesions and in both experimental and clinical cholangiocarcinomas (Yoon et al. 2010).

Biomarkers of NADPH System

NADPH is the principal cofactor used as an electron donor in various reactions. NADPH is primarily synthesized via the pentose phosphate pathway, which is the main pathway for the synthesis of ribose required for the production of DNA and RNA. NADPH contributes to cell survival via modulation of the cellular pH and redox status (Furuta et al. 2010). In fact, NADPH is tightly associated with the glutathione and thioredoxin systems (Penney and Roy 2013) in the maintenance of the oxidation-reduction balance. Additionally, NADPH is used as a cofactor by a variety of enzymes that mediate oxidation-reduction reactions as part of the phase II antioxidant response.

Glucose-6-Phosphate Dehydrogenase

The enzyme glucose-6-phosphate dehydrogenase (G6PD) plays a fundamental role in the synthesis of ribose-5-phosphate and NADPH and is essential for the antioxidant response (Furuta et al. 2010). The presence of G6PD in solid

tumors is a biomarker due to its critical role for the synthesis of nucleic acids and maintenance of the cellular redox status. In fact, the complete pentose phosphate pathway is overexpressed in hepatocellular carcinomas compared with non-tumor tissues (Perez-Carreon et al. 2006; Shimizu et al. 2014). Future studies should focus on understanding how G6PD participates with other enzymes such as NADP transhydrogenase, NADP-dependent malate, and NADP-dependent isocitric dehydrogenase in the production of NADPH within hepatic tumors.

Heme Oxygenase-1

Heme oxygenase-1 (HMOX-1) is a microsomal enzyme that is highly inducible by various stimuli, such as UV light, reactive species, and hypoxia (Yin et al. 2012). HMOX-1 enzymes catalyze the rate-limiting reaction in the metabolism of heme groups, giving rise to biliverdin, iron, and carbon monoxide. The enzyme and its resulting metabolites present antioxidant, anti-inflammatory, anti-apoptotic, and immunomodulating functions (Noh et al. 2013). Therefore, HMOX-1 enzymes participate in the process of redox control by maintaining cellular homeostasis. The expression of HMOX-1 in liver cancer is a biomarker associated with increased resistance to treatment and increased invasiveness. Expression of the HMOX-1 enzyme is not limited to tumor tissue, as it is also expressed in adjacent and cirrhotic tissue (Cheng et al. 2015).

NADPH Quinone Oxidoreductase 1

NAD(P)H dehydrogenase [quinone] 1 (NQO1) is a cytosolic enzyme that uses NADPH to directly reduce quinones and hydroquinones (Lin et al. 2014). Additionally, NQO1 participates in the metabolism of superoxide and the maintenance of endogenous vitamins (Yang et al. 2014). Some of the functions of NQO1 enzymes include protection against cytotoxic and carcinogenic quinone insults and protection from oxidative stress. The NQO1 enzyme interacts directly with the NF-kB, NFE2L2, and p53 pathways (Wakai et al. 2011; Jamshidi et al. 2012). Expression of NQO1 in tumors favors resistance to treatment and is considered a biomarker of a poor prognosis in patients.

In patients with cholangiocarcinoma, overexpression of NQO1 has been reported in late and differentiated stages; however, NQO1 expression is weak in tumors in intermediate and early differentiated stages. How the expression of this enzyme affects the prognosis of patients with cholangiocarcinomas remains under study (Wakai et al. 2011; Buranrat et al. 2012). Overexpression of NQO1 has also been described in cases of hepatocellular carcinoma in both experimental models and patients (Petrelli et al. 2014).

Prostaglandin Reductase

The prostaglandin reductase (PTGR) enzymes catalyze NADPH-dependent oxidoreductive reactions. PTGRs are primarily involved in the catabolism of leukotrienes, prostaglandins, aldehydes, and ketones. Additionally, PTGRs catabolize products generated during oxidative stress, as is the case of alpha,beta-unsaturated 4-HNE, produced via lipid peroxidation in cells (Dick et al. 2001). In solid tumors, the expression of PTGR is associated with the modulation of 4-HNE and prevention of 4-HNE-induced cell death.

In experimental models and clinical samples of hepatocellular carcinomas, PTGR1 overexpression is present as an early biomarker (Sanchez-Rodriguez et al. 2014). Moreover, expression of PTGR1 is included in the list of genes that has been associated with progenitor cells for hepatocellular carcinoma (Ho et al. 2012).

Carbonyl Reductase 1

Carbonyl reductase 1 (CBR1) is a monomeric NADPH-dependent cytosolic enzyme that catalyzes the metabolism of compounds carrying a carbonyl group, such as antibiotics, antitumor drugs, and prostaglandins (Murakami et al. 2012). CBR1 is also involved in the metabolism of products of lipid peroxidation reactions, such as 4-oxonon-2-enal, a reactive species that modifies proteins and DNA. The role of CBR1 in carcinogenesis is not currently clear; however, CBR1 is a biomarker overexpressed in hepatocellular carcinomas, and its overexpression appears to correlate with the stage of progression. CBR1 is thought to provide protection against reactive species in cells (Tak et al. 2011).

Aldo-Keto Reductase

The superfamily of aldo-keto reductase enzymes (AKRs) includes 15 genetically related families. These enzymes participate in the reduction of aldehydes and ketones, which are reactions that depend on NADPH as a cofactor (Wang et al. 2010). Additionally, these enzymes contribute to the metabolism of steroids, carbohydrates, and prostaglandins (Endo et al. 2010). AKRs are also thought to play a role in the cellular processes of proliferation and angiogenesis (Chellappa et al. 2012). The AKR1 family is more consistently expressed in neoplastic cells, and these proteins are biomarkers associated with a poor prognosis and greater invasiveness in cases involving solid tumors. In liver cancer, cholangiocarcinomas, and hepatocellular carcinoma, overexpression of AKR1B10 has been confirmed in clinical samples at early and intermediate

stages relative to healthy tissue, while AKR1B10 expression is lost in advanced tumor stages (Heringlake et al. 2010). In experimental models of hepatocellular carcinoma, both AKR7A3 and AKR1B1 have been found to be overexpressed at early stages in preneoplastic tissue in comparison with healthy tissue (Albrethsen et al. 2011).

Metallothionein as Biomarkers

Metallothioneins (MTs) belong to a family of low-molecular-weight proteins that are expressed in response to stress. These proteins exhibit conserved cysteine-rich domains that are able to bind to metals such as zinc, copper, cadmium, mercury, and platinum, thus playing a role in metal homeostasis (Werynska et al. 2013). In addition, MT proteins efficiently bind to reactive species. Various agents, such as hormones, cytotoxic agents, and inflammatory cytokines, induce MT expression. These proteins participate in cellular protection against UV radiation, modulation of reactive species, and protection against chemotherapeutic agents (Gumulec et al. 2014). The expression of MTs is elevated in solid tumors, though the exact levels are isoform specific. Moreover, their expression is a biomarker that tends to unfavorably affect the response to chemotherapy, as these proteins critically influence cell proliferation.

In papillary thyroid carcinoma and breast, oral, colon, kidney, and lung cancers, the MT1 and MT2 isoforms are overexpressed. Reports concerning the expression levels in HCC are inconsistent, but it is known that the MT1H and MT1G subtypes may participate as suppressors of proliferation (Liu et al. 2009; Gumulec et al. 2014). The activation and silencing mechanisms of these proteins in liver cancer are of particular interest; for example, in colorectal cancer, the expression of MT1G, MT1F, MT1H, MT1M, MT1X, and MT2A is lost during the transition from a normal mucosa to a tumor. The main mechanism thought to play a role in reducing expression of these isoforms is silencing via DNA hypermethylation (Arriaga et al. 2012).

Potential Applications to Prognosis, Other Diseases, or Conditions

The various enzymes of the antioxidant response are overexpressed from early stages of liver carcinogenesis and often are indicative of response to chemotherapy, cancer diagnosis, prognosis, and recurrence. Considering the high heterogeneity of tumors, the use of molecular diagnosis such as expression profile of antioxidant enzymes could contribute to better classification of tumors and precise diagnosis. This application could be extended for other cancers such as kidney, lung, ovarian, brain, and colon (Hanigan et al. 1994; Bartsch et al. 2002; Liu et al. 2009; Pastor et al. 2013; Gumulec et al. 2014), where high protein expression of antioxidant response enzymes has been reported also; this profile has been associated with resistance to cell death, increased proliferation, resistance to chemotherapy treatment, and metastasis. The antioxidant proteins as

biomarkers of tumors may allow chemical design for pharmacological treatments, for example, antitumor agents such as mitomycin C and the acylfulvenes, which are activated by enzymatic bioreduction. Thus, molecular diagnosis using antioxidant biomarkers will contribute to a better personalized therapy for cancer patients.

The antioxidant enzymes such as GGT, SOD, and PRX1 were measured with high levels in diseases such as coronary astery disease (Paolicchi et al. 2004; Rivollier et al. 2006; Franzini et al. 2009). Additionally, neurodegenerative diseases such as Huntington, Parkinson, Alzheimer, and amyotrophic lateral sclerosis are closely related with oxidative stress, so the roles of the antioxidant response and the use of related proteins to the redox metabolism have been considered as a therapeutic target, and they are subject of current research (Gan and Johnson 2014).

Conclusion and Future Biomarker Research

The antioxidant enzymes and antioxidant pathways are known to be present and active in tumor cells, and we could use them as biomarkers for diagnosis, recurrence, and prognosis associated to liver carcinogenesis. Furthermore the enzymatic redox capabilities of these kinds of biomarkers could be used as target for chemotherapy of HCC. Most studies show that even though tumors overexpress a variety of antioxidant enzymes, high levels of reactive species are still present; this is more complicated considering the difficulty to measure the exact oxidation-reduction balance in tumor cells, due to their heterogeneity and asynchrony. The high level of GSH and the reduced state of cancer cells, which are conditions that would favor cell proliferation and the cell death evasion, may contribute to tumor burden (Bobko et al. 2012). The biomarker research on antioxidant response may help to understand whether oxidative stress is present or absent in tumor cells or whether tumor cells exhibit a completely new redox equilibrium status (Droge 2002) that may allow a cellular reduced state or tolerance to high levels of reactive species. Considering the tumor microenvironment complexity of the biomarker research, it is also important to explore the differences in the redox potential between neoplastic cells and normal surrounding cells. Moreover, the mechanisms responsible for redox adaptation in tumor cells merit clarification; also several studies are necessary to determine a gene expression profile of antioxidant response genes in liver tumors. This profile of biomarkers of the antioxidant response may contribute to better diagnosis and classification of tumors in liver carcinogenesis.

Summary Points

- This chapter focuses on biomarkers of antioxidant response in the liver in particular a focus on cancer.
- Antioxidant response is the cellular mechanism that counteracts oxidative stress.

- Oxidative stress is a measurable index. But very often the stress response is measurable after the initiation of cellular events.
- Biomarkers, particularly those related to genes or proteins, have the potential to be measurable before cellular events.
- Biomarkers of the antioxidant response include three main protein-metabolite systems: glutathione, thioredoxins, and nicotinamide adenine dinucleotide phosphate.
- Several proteins involved in antioxidant response are overexpressed in liver tumors, and they are associated with diagnosis, prognosis, and response to therapy.
- The antioxidant response may contribute to tumoral development as an adaptive response to maintain a reduced redox balance.
- These include glutathione reductase, glutathione S-transferase P, gamma-glutamyl transferase, glucose-6-phosphate dehydrogenase, thioredoxin reductase, NAD(P)H dehydrogenase [quinone] 1, and prostaglandin reductase 1.

References

Albrethsen J, Miller LM, Novikoff PM, Angeletti RH. Gel-based proteomics of liver cancer progression in rat. Biochim Biophys Acta. 2011;1814(10):1367–76.

Arriaga JM, Levy EM, Bravo AI, Bayo SM, Amat M, Aris M, Hannois A, Bruno L, Roberti MP, Loria FS, Pairola A, Huertas E, Mordoh J, Bianchini M. Metallothionein expression in colorectal cancer: relevance of different isoforms for tumor progression and patient survival. Hum Pathol. 2012;43(2):197–208.

Backos DS, Fritz KS, McArthur DG, Kepa JK, Donson AM, Petersen DR, Foreman NK, Franklin CC, Reigan P. Glycation of glutamate cysteine ligase by 2-deoxy-d-ribose and its potential impact on chemoresistance in glioblastoma. Neurochem Res. 2013;38(9):1838–49.

Bae YS, Oh H, Rhee SG, Yoo YD. Regulation of reactive oxygen species generation in cell signaling. Mol Cells. 2011;32(6):491–509.

Bartsch H, Nair J, Owen RW. Exocyclic DNA adducts as oxidative stress markers in colon carcinogenesis: potential role of lipid peroxidation, dietary fat and antioxidants. Biol Chem. 2002;383(6):915–21.

Bobko AA, Eubank TD, Voorhees JL, Efimova OV, Kirilyuk IA, Petryakov S, Trofimiov DG, Marsh CB, Zweier JL, Grigor'ev IA, Samouilov A, Khramtsov VV. In vivo monitoring of pH, redox status, and glutathione using L-band EPR for assessment of therapeutic effectiveness in solid tumors. Magn Reson Med. 2012;67(6):1827–36.

Buranrat B, Chau-in S, Prawan A, Puapairoj A, Zeekpudsa P, Kukongviriyapan V. NQO1 expression correlates with cholangiocarcinoma prognosis. Asian Pac J Cancer Prev. 2012;13 (Suppl):131–6.

Chellappa K, Jankova L, Schnabl JM, Pan S, Brelivet Y, Fung CL, Chan C, Dent OF, Clarke SJ, Robertson GR, Sladek FM. Src tyrosine kinase phosphorylation of nuclear receptor HNF4alpha correlates with isoform-specific loss of HNF4alpha in human colon cancer. Proc Natl Acad Sci U S A. 2012;109(7):2302–7.

Chen ZS, Tiwari AK. Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases. FEBS J. 2011;278(18):3226–45.

Cheng ML, Lu YF, Chen H, Shen ZY, Liu J. Liver expression of Nrf2-related genes in different liver diseases. Hepatobiliary Pancreat Dis Int. 2015;14(5):485–91.

Circu ML, Aw TY. Glutathione and apoptosis. Free Radic Res. 2008;42(8):689–706.

Colak D, Chishti MA, Al-Bakheet AB, Al-Qahtani A, Shoukri MM, Goyns MH, Ozand PT, Quackenbush J, Park BH, Kaya N. Integrative and comparative genomics analysis of early hepatocellular carcinoma differentiated from liver regeneration in young and old. Mol Cancer. 2010;9:146.

Corti A, Franzini M, Paolicchi A, Pompella A. Gamma-glutamyltransferase of cancer cells at the crossroads of tumor progression, drug resistance and drug targeting. Anticancer Res. 2010; 30(4):1169–81.

Czeczot H, Scibior D, Skrzycki M, Podsiad M. Glutathione and GSH-dependent enzymes in patients with liver cirrhosis and hepatocellular carcinoma. Acta Biochim Pol. 2006; 53(1):237–42.

Dalleau S, Baradat M, Gueraud F, Huc L. Cell death and diseases related to oxidative stress: 4-hydroxynonenal (HNE) in the balance. Cell Death Differ. 2013;20(12):1615–30.

Dick RA, Kwak MK, Sutter TR, Kensler TW. Antioxidative function and substrate specificity of NAD(P)H-dependent alkenal/one oxidoreductase. A new role for leukotriene B4 12-hydroxydehydrogenase/15-oxoprostaglandin 13-reductase. J Biol Chem. 2001; 276(44):40803–10.

Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82(1):47–95.

El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology. 2007;132(7):2557–76.

Endo S, Matsunaga T, Soda M, Tajima K, Zhao HT, El-Kabbani O, Hara A. Selective inhibition of the tumor marker AKR1B10 by antiinflammatory N-phenylanthranilic acids and glycyrrhetic acid. Biol Pharm Bull. 2010;33(5):886–90.

Franco R, Cidlowski JA. Glutathione efflux and cell death. Antioxid Redox Signal. 2012; 17(12):1694–713.

Franzini M, Corti A, Martinelli B, Del Corso A, Emdin M, Parenti GF, Glauber M, Pompella A, Paolicchi A. Gamma-glutamyltransferase activity in human atherosclerotic plaques – biochemical similarities with the circulating enzyme. Atherosclerosis. 2009;202(1):119–27.

Fu TY, Hou YY, Chu ST, Liu CF, Huang CH, Chen HC, Hsiao M, Lu PJ, Wang JS, Ger LP. Manganese superoxide dismutase and glutathione peroxidase as prognostic markers in patients with buccal mucosal squamous cell carcinomas. Head Neck. 2011; 33(11):1606–15.

Furuta E, Okuda H, Kobayashi A, Watabe K. Metabolic genes in cancer: their roles in tumor progression and clinical implications. Biochim Biophys Acta. 2010;1805(2):141–52.

Gan L, Johnson JA. Oxidative damage and the Nrf2-ARE pathway in neurodegenerative diseases. Biochim Biophys Acta. 2014;1842(8):1208–18.

Gumulec J, Raudenska M, Adam V, Kizek R, Masarik M. Metallothionein – immunohistochemical cancer biomarker: a meta-analysis. PLoS One. 2014;9(1):e85346.

Halliwell B, Gutteridge JM. Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. Lancet. 1984;1(8391):1396–7.

Hanigan MH, Frierson Jr HF, Brown JE, Lovell MA, Taylor PT. Human ovarian tumors express gamma-glutamyl transpeptidase. Cancer Res. 1994;54(1):286–90.

Heringlake S, Hofdmann M, Fiebeler A, Manns MP, Schmiegel W, Tannapfel A. Identification and expression analysis of the aldo-ketoreductase1-B10 gene in primary malignant liver tumours. J Hepatol. 2010;52(2):220–7.

Ho DW, Yang ZF, Yi K, Lam CT, Ng MN, Yu WC, Lau J, Wan T, Wang X, Yan Z, Liu H, Zhang Y, Fan ST. Gene expression profiling of liver cancer stem cells by RNA-sequencing. PLoS One. 2012;7(5):e37159.

Jamshidi M, Bartkova J, Greco D, Tommiska J, Fagerholm R, Aittomaki K, Mattson J, Villman K, Vrtel R, Lukas J, Heikkila P, Blomqvist C, Bartek J, Nevanlinna H. NQO1 expression correlates inversely with NFkappaB activation in human breast cancer. Breast Cancer Res Treat. 2012; 132(3):955–68.

Kekec Y, Paydas S, Tuli A, Zorludemir S, Sakman G, Seydaoglu G. Antioxidant enzyme levels in cases with gastrointesinal cancer. Eur J Intern Med. 2009;20(4):403–6.

Kim S, Jung WH, Koo JS. Differences in autophagy-related activity by molecular subtype in triple-negative breast cancer. Tumour Biol. 2012;33(5):1681–94.

Lin L, Qin Y, Jin T, Liu S, Zhang S, Shen X, Lin Z. Significance of NQO1 overexpression for prognostic evaluation of gastric adenocarcinoma. Exp Mol Pathol. 2014;96(2):200–5.

Liu ZM, Hasselt CA, Song FZ, Vlantis AC, Cherian MG, Koropatnick J, Chen GG. Expression of functional metallothionein isoforms in papillary thyroid cancer. Mol Cell Endocrinol. 2009; 302(1):92–8.

Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet. 2003;362(9399):1907–17.

Marra M, Sordelli IM, Lombardi A, Lamberti M, Tarantino L, Giudice A, Stiuso P, Abbruzzese A, Sperlongano R, Accardo M, Agresti M, Caraglia M, Sperlongano P. Molecular targets and oxidative stress biomarkers in hepatocellular carcinoma: an overview. J Transl Med. 2011;9:171.

Matos JM, Witzmann FA, Cummings OW, Schmidt CM. A pilot study of proteomic profiles of human hepatocellular carcinoma in the United States. J Surg Res. 2009;155(2):237–43.

Mougiakakos D, Okita R, Ando T, Durr C, Gadiot J, Ichikawa J, Zeiser R, Blank C, Johansson CC, Kiessling R. High expression of GCLC is associated with malignant melanoma of low oxidative phenotype and predicts a better prognosis. J Mol Med (Berl). 2012;90(8):935–44.

Murakami A, Yakabe K, Yoshidomi K, Sueoka K, Nawata S, Yokoyama Y, Tsuchida S, Al-Mulla F, Sugino N. Decreased carbonyl reductase 1 expression promotes malignant behaviours by induction of epithelial mesenchymal transition and its clinical significance. Cancer Lett. 2012;323(1):69–76.

Murtas D, Piras F, Minerba L, Ugalde J, Floris C, Maxia C, Demurtas P, Perra MT, Sirigu P. Nuclear 8-hydroxy-2′-deoxyguanosine as survival biomarker in patients with cutaneous melanoma. Oncol Rep. 2010;23(2):329–35.

Noh SJ, Bae JS, Jamiyandorj U, Park HS, Kwon KS, Jung SH, Youn HJ, Lee H, Park BH, Chung MJ, Moon WS, Kang MJ, Jang KY. Expression of nerve growth factor and heme oxygenase-1 predict poor survival of breast carcinoma patients. BMC Cancer. 2013;13:516.

Noike T, Miwa S, Soeda J, Kobayashi A, Miyagawa S. Increased expression of thioredoxin-1, vascular endothelial growth factor, and redox factor-1 is associated with poor prognosis in patients with liver metastasis from colorectal cancer. Hum Pathol. 2008;39(2):201–8.

Ooi A, Wong JC, Petillo D, Roossien D, Perrier-Trudova V, Whitten D, Min BW, Tan MH, Zhang Z, Yang XJ, Zhou M, Gardie B, Molinie V, Richard S, Tan PH, Teh BT, Furge KA. An antioxidant response phenotype shared between hereditary and sporadic type 2 papillary renal cell carcinoma. Cancer Cell. 2011;20(4):511–23.

Oyagbemi AA, Azeez OI, Saba AB. Interactions between reactive oxygen species and cancer: the roles of natural dietary antioxidants and their molecular mechanisms of action. Asian Pac J Cancer Prev. 2009;10(4):535–44.

Pallardo FV, Markovic J, Garcia JL, Vina J. Role of nuclear glutathione as a key regulator of cell proliferation. Mol Aspects Med. 2009;30(1–2):77–85.

Paolicchi A, Emdin M, Ghliozeni E, Ciancia E, Passino C, Popoff G, Pompella A. Images in cardiovascular medicine. Human atherosclerotic plaques contain gamma-glutamyl transpeptidase enzyme activity. Circulation. 2004;109(11):1440.

Pastor MD, Nogal A, Molina-Pinelo S, Melendez R, Salinas A, Gonzalez De la Pena M, Martin-Juan J, Corral J, Garcia-Carbonero R, Carnero A, Paz-Ares L. Identification of proteomic signatures associated with lung cancer and COPD. J Proteomics. 2013;89:227–37.

Penney RB, Roy D. Thioredoxin-mediated redox regulation of resistance to endocrine therapy in breast cancer. Biochim Biophys Acta. 2013;1836(1):60–79.

Perez-Carreon JI, Lopez-Garcia C, Fattel-Fazenda S, Arce-Popoca E, Aleman-Lazarini L, Hernandez-Garcia S, Le Berre V, Sokol S, Francois JM, Villa-Trevino S. Gene expression profile related to the progression of preneoplastic nodules toward hepatocellular carcinoma in rats. Neoplasia. 2006;8(5):373–83.

Petrelli A, Perra A, Cora D, Sulas P, Menegon S, Manca C, Migliore C, Kowalik MA, Ledda-Columbano GM, Giordano S, Columbano A. MicroRNA/gene profiling unveils early

molecular changes and nuclear factor erythroid related factor 2 (NRF2) activation in a rat model recapitulating human hepatocellular carcinoma (HCC). Hepatology. 2014; 59(1):228–41.

Reddy NM, Kleeberger SR, Bream JH, Fallon PG, Kensler TW, Yamamoto M, Reddy SP. Genetic disruption of the Nrf2 compromises cell-cycle progression by impairing GSH-induced redox signaling. Oncogene. 2008;27(44):5821–32.

Reszka E. Selenoproteins in bladder cancer. Clin Chim Acta. 2012;413(9–10):847–54.

Rivollier A, Perrin-Cocon L, Luche S, Diemer H, Strub JM, Hanau D, van Dorsselaer A, Lotteau V, Rabourdin-Combe C, Rabilloud T, Servet-Delprat C. High expression of antioxidant proteins in dendritic cells: possible implications in atherosclerosis. Mol Cell Proteomics. 2006; 5(4):726–36.

Saeidnia S, Abdollahi M. Antioxidants: friends or foe in prevention or treatment of cancer: the debate of the century. Toxicol Appl Pharmacol. 2013;271(1):49–63.

Sainz RM, Lombo F, Mayo JC. Radical decisions in cancer: redox control of cell growth and death. Cancers (Basel). 2012;4(2):442–74.

Sanchez-Perez Y, Carrasco-Legleu C, Garcia-Cuellar C, Perez-Carreon J, Hernandez-Garcia S, Salcido-Neyoy M, Aleman-Lazarini L, Villa-Trevino S. Oxidative stress in carcinogenesis. Correlation between lipid peroxidation and induction of preneoplastic lesions in rat hepatocarcinogenesis. Cancer Lett. 2005;217(1):25–32.

Sanchez-Rodriguez R, Torres-Mena JE, De-la-Luz-Cruz M, Bernal-Ramos GA, Villa-Trevino S, Chagoya-Hazas V, Landero-Lopez L, Garcia-Roman R, Rouimi P, Del-Pozo-Yauner L, Melendez-Zajgla J, Perez-Carreon JI. Increased expression of prostaglandin reductase 1 in hepatocellular carcinomas from clinical cases and experimental tumors in rats. Int J Biochem Cell Biol. 2014;53:186–94.

Sayin VI, Ibrahim MX, Larsson E, Nilsson JA, Lindahl P, Bergo MO. Antioxidants accelerate lung cancer progression in mice. Sci Transl Med. 2014;6(221):221ra215.

Sengupta R, Holmgren A. The role of thioredoxin in the regulation of cellular processes by S-nitrosylation. Biochim Biophys Acta. 2012;1820(6):689–700.

Shimizu T, Inoue KI, Hachiya H, Shibuya N, Shimoda M, Kubota K. Frequent alteration of the protein synthesis of enzymes for glucose metabolism in hepatocellular carcinomas. J Gastroenterol. 2014;49(9):1324–1332. PMCID: PMC4156784.

Sorokina LV, Solyanik GI, Pyatchanina TV. The evaluation of prooxidant and antioxidant state of two variants of lewis lung carcinoma: a comparative study. Exp Oncol. 2010;32(4):249–53.

Sun QK, Zhu JY, Wang W, Lv Y, Zhou HC, Yu JH, Xu GL, Ma JL, Zhong W, Jia WD. Diagnostic and prognostic significance of peroxiredoxin 1 expression in human hepatocellular carcinoma. Med Oncol. 2014;31(1):786.

Suzuki S, Pitchakarn P, Ogawa K, Naiki-Ito A, Chewonarin T, Punfa W, Asamoto M, Shirai T, Takahashi S. Expression of glutathione peroxidase 2 is associated with not only early hepatocarcinogenesis but also late stage metastasis. Toxicology. 2013;311(3):115–23.

Tak E, Lee S, Lee J, Rashid MA, Kim YW, Park JH, Park WS, Shokat KM, Ha J, Kim SS. Human carbonyl reductase 1 upregulated by hypoxia renders resistance to apoptosis in hepatocellular carcinoma cells. J Hepatol. 2011;54(2):328–39.

Uchida M, Sugaya M, Kanamaru T, Hisatomi H. Alternative RNA splicing in expression of the glutathione synthetase gene in human cells. Mol Biol Rep. 2010;37(4):2105–9.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39(1): 44–84.

Wakai T, Shirai Y, Sakata J, Matsuda Y, Korita PV, Takamura M, Ajioka Y, Hatakeyama K. Prognostic significance of NQO1 expression in intrahepatic cholangiocarcinoma. Int J Clin Exp Pathol. 2011;4(4):363–70.

Wang MY, Liehr JG. Lipid hydroperoxide-induced endogenous DNA adducts in hamsters: possible mechanism of lipid hydroperoxide-mediated carcinogenesis. Arch Biochem Biophys. 1995; 316(1):38–46.

Wang X, Chorley BN, Pittman GS, Kleeberger SR, Brothers 2nd J, Liu G, Spira A, Bell DA. Genetic variation and antioxidant response gene expression in the bronchial airway epithelium of smokers at risk for lung cancer. PLoS One. 2010;5(8):e11934.

Werynska B, Pula B, Muszczynska-Bernhard B, Gomulkiewicz A, Piotrowska A, Prus R, Podhorska-Okolow M, Jankowska R, Dziegiel P. Metallothionein 1F and 2A overexpression predicts poor outcome of non-small cell lung cancer patients. Exp Mol Pathol. 2013; 94(1):301–8.

Yang Y, Zhang Y, Wu Q, Cui X, Lin Z, Liu S, Chen L. Clinical implications of high NQO1 expression in breast cancers. J Exp Clin Cancer Res. 2014;33:14.

Yin Y, Liu Q, Wang B, Chen G, Xu L, Zhou H. Expression and function of heme oxygenase-1 in human gastric cancer. Exp Biol Med (Maywood). 2012;237(4):362–71.

Yoon BI, Kim YH, Yi JY, Kang MS, Jang JJ, Joo KH, Kim Y, McHugh Law J, Kim DY. Expression of thioredoxin during progression of hamster and human cholangiocarcinoma. Cancer Sci. 2010;101(1):281–8.

Yu Z, Peng S, Hong-Ming P, Kai-Feng W. Expression of multi-drug resistance-related genes MDR3 and MRP as prognostic factors in clinical liver cancer patients. Hepatogastroenterology. 2012;59(117):1556–9.

Zhang H, Forman HJ. Glutathione synthesis and its role in redox signaling. Semin Cell Dev Biol. 2012;23(7):722–8.

Zuniga-Garcia V, Chavez-Lopez Mde G, Quintanar-Jurado V, Gabino-Lopez NB, Hernandez-Gallegos E, Soriano-Rosas J, Perez-Carreon JI, Camacho J. Differential expression of ion channels and transporters during hepatocellular carcinoma development. Dig Dis Sci. 2015; 60(8):2373–83.

microRNA-155 and microRNA-196b in Hepatitis C Virus Infection

39

Ewelina Kałużna

Contents

Abstract

microRNAs (miRNAs) are a class of small, endogenous, noncoding RNAs that play a significant role in the regulation of both physiological and pathological processes. Growing evidence suggests that they are also involved in hepatitis C

E. Kałużna (✉)
Department of Molecular Pathology, Institute of Human Genetics, Polish Academy of Sciences, Poznań, Poland
e-mail: ewelina.kaluzna@o2.pl; ewelinak@man.poznan.pl

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_16

virus (HCV) infection. It has been shown that miRNAs may both directly and indirectly affect the HCV life cycle, as well as the biological pathways crucial for the development of hepatitis C and HCV-related liver diseases. Hepatitis C is a growing health problem worldwide. It is estimated that approximately 3% of the global population is infected with HCV, and about 350–500,000 people die each year from HCV-related liver disorders, such as cirrhosis and hepatocellular carcinoma. There is, therefore, a strong need to identify markers that allow the monitoring of chronic hepatitis C (CHC) progression, as well as to identify patients that will not respond to treatment. This chapter has summarized recent studies on the role of two selected miRNAs – miRNA-155 and miRNA-196b – in HCV infection and CHC. It discusses the significance and involvement of these molecules in regulating the HCV life cycle, the development of HCV infection and HCV-related liver diseases, as well as their influence on the course of CHC. Special emphasis has been given to their potential applications as diagnostic, prognostic, and predictive biomarkers and as targets of novel antiviral therapies.

Keywords

miRNA • miRNA-155 • miRNA-196 • Hepatitis C virus • HCV infection • Chronic hepatitis C

List of Abbreviations

Ago	Argonaute
aGVHD	Acute graft-versus-host disease
ALT	Alanine aminotransferase
ANXA1	Annexin A1
APC	Adenomatous polyposis coli
AST	Aspartate aminotransferase
AUC	Area under the receiver-operating characteristic curve
BACH1	BTB and CNC homology 1 and basic leucine zipper transcription factor 1
BIC	B-cell integration cluster
C/EBPβ	CCAAT/enhancer-binding protein beta
CHC	Chronic hepatitis C
DAAs	Direct-acting antivirals
ECM	Extracellular matrix
Exp5	Exportin-5
FAS	Fas cell surface death receptor
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCV NS	HCV nonstructural protein
HMAGA2	Nuclear architectural factor
HMOX1	Heme oxygenase 1
IFN	Interferon

IGF2BP1	Insulin-like growth factor 2 RNA-binding protein 1
IKKs	IkB kinases
IL	Interleukin
ISGs	IFN-stimulated genes
JAK-STAT	Janus kinase-signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
kb	Kilobases
LPS	Lipopolysaccharide
LT	Liver transplantation
MAPK	Mitogen-activated protein kinase
MEIS1	Meis homeobox 1
MiRNAs	microRNAs
MLL	Mixed lineage leukemia
mRNA	Messenger RNA
NCR	Noncoding region
NF-kB	Nuclear factor kappa B
NK cells	Natural killer cells
NRs	Nonresponders
nt	Nucleotides
ORF	Open reading frame
PBMCs	Peripheral blood mononuclear cells
peg-INF-α + RBV	Pegylated interferon-α and ribavirin
poly-I:C	Polyriboinosinic-polyribocytidilic acid
Pre-miRNA	Precursory miRNA
Pri-miRNA	Primary miRNA
RA	Rheumatoid arthritis
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
SVR	Sustained virologic response
TLRs	Toll-like receptors
TNBC	Triple-negative breast cancer
TNF	Tumor necrosis factor
TNM	Classification of malignant tumors: T (tumor), N (nodes), M (metastasis)
UTR	Untranslated region

Key Facts of Chronic Hepatitis C

- Chronic hepatitis C (CHC) is a liver disease caused by the blood-borne hepatitis C virus (HCV).
- Approximately 130–150 million people are infected with HCV worldwide.
- CHC is the main cause of liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC).

- Three hundred to 500,000 people die each year from hepatitis C-related liver diseases.
- There is no vaccine for hepatitis C.
- Currently available antiviral treatment is successful in 50–90% of cases, depending on the treatment used.
- There is a major need for biomarkers that allow us to monitor and predict the progress of CHC as well as the effectiveness of antiviral treatment

Definition of Words and Terms

Antigenomic HCV RNA strand	A replicative intermediate RNA molecule of negative polarity.
HCV genotype	Genetic heterogeneity among different HCV isolates.
HCV infectivity	The number of complete, mature HCV particles, capable of infecting, derived from one RNA molecule.
HCV quasispecies	Genetic variants within one isolate.
HCV subtype	Closely related isolates within one genotype.
Liver cirrhosis	Architectural distortion of the liver, including diffuse parenchymal nodularity, fibrosis, and vascular changes of variable severity.
Liver fibrosis	Imbalance between the production and degradation of the extracellular matrix that results in progressive accumulation of its components.
Metastasis	The formation of progressively growing secondary tumor foci at sites discontinuous from the primary lesion.
Nucleotide similarity (%)	The percentage of sequence identity within the full genome.
Pegylated IFN	An IFN molecule modified through the addition of polyethylene glycol in order to improve IFN pharmacokinetic properties.
Sustained virologic response (SVR)	Lack of HCV RNA in the serum 6 months after the completion of antiviral therapy: complete virus eradication.
Virion	A single, complete viral particle, infecting the cell and capable of survival outside it.

Introduction

Hepatitis C is a liver disease caused by the blood-borne hepatitis C virus (HCV) and is considered a major public health problem. It is estimated that approximately 130–150 million people are infected with HCV worldwide. HCV infection may lead to acute or

chronic liver hepatitis, with the vast majority of infections becoming chronic. Chronic hepatitis C (CHC) is thought to be a major cause of progressive fibrosis and cirrhosis of the liver, leading to the development of hepatocellular carcinoma (HCC) (according to the World Health Organization 2016). One of the approaches to the treatment of CHC is pegylated interferon-alpha in combination with ribavirin (a two-drug therapy, peg-IFN-α + RBV). The treatment efficacy with two-drug therapy largely depends on HCV genotype. It is estimated that for patients infected with HCV genotype 1, sustained response is achieved in about 40%, but for patients with genotype 2, 3, 5, or 6 in approximately 80%. In the treatment of HCV genotype 1, triple therapy may be employed: peg-IFN-α + RBV with viral protease or polymerase inhibitors. Triple therapy increases the cure rate of previously untreated patients up to about 70%, but in the case of patient previously unsuccessfully treated with two-drug therapy, triple treatment is effective only in about 50%. Newly developed IFN-free treatment seems to be the best approach for CHC patients, due to the high cure rate (80–95%) and a lack of IFN-associated side effects (EASL Recommendations on Treatment of Hepatitis C 2015). However, IFN-free therapy is extremely expensive, which makes it difficult to obtain even for patients in high-income countries. Moreover, there is a risk of generating drug-resistant HCV strains.

The mechanism underlying the elimination of the virus and its persistence even after antiviral therapy has been completed has not been fully understood and explained. There is a significant need to identify markers that allow the monitoring of the progress of CHC, as well as the identification of patients who are unlikely to respond to treatment. These markers could help to identify patients with higher risk of HCV-related life-threatening liver diseases, reduce the exposure of patients to side effects, and reduce public costs, hence the interest of scientists in small, noncoding regulatory RNA molecules, called microRNAs (miRNAs or miRs). Growing evidence suggests these molecules have a significant impact on the HCV life cycle and the course of CHC.

This chapter aims to discuss the miRNAs involved in controlling HCV infection, particularly miRNA-155 and miRNA-196b. Emphasis has been given to their potential usefulness for making prognoses as to the course of CHC and the effectiveness of treatment, as well as their potential application as novel therapeutic targets.

Hepatitis C

Hepatitis C Virus

The hepatitis C virus was discovered by molecular cloning by Choo et al. (1989). The researchers found unknown viral antigens in the plasma of a chimpanzee infected with non-A, non-B viral hepatitis by contaminated factor XIII concentrate. Since then, HCV has become the subject of intensive research. HCV has been classified as belonging to the family *Flaviviridae* and the genus *Hepacivirus*. The HCV genome consists of positive-sense single-stranded RNA, with a length of approximately 9,600 nucleotides (nt) (Choo et al. 1991). It is organized into the 5′ noncoding region (NCR) of 341 bases and has a single open reading frame (ORF) of about 9,050 nt and a 3′

NCR of 27 nt. Flanked regions at the 3′ and 5′ ends contain important sequence and structural elements essential for HCV translation and RNA replication (Friebe and Bartenschlager 2002). The ORF encodes a polyprotein of about 3,011 amino acids, which is proteolytically processed into three mature structural (C, E1 and E2) and seven nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (Fig. 1; Simmonds 2004; Bartenschlager et al. 2011). The replication and translation of HCV occur in the cytoplasm of hepatocytes, but HCV is not directly cytopathic. The translation initiation depends on internal ribosome entry sites (IRESs), which bind directly to the 40S ribosomal subunit (Pestova et al. 1998).

The sequencing of HCV isolates from various parts of the world reveals differences in about 31–33% of nucleotide sites (Simmonds et al. 2005). Based on this genetic variability, HCV has been divided into seven main genetic lineages: the genotypes are numbered 1–7 (although some experts state that there may even be 11 HCV genotypes) with a nucleotide similarity of 66–67%. The genotypes are further divided into subtypes and the subtypes into quasispecies, with nucleotide similarities of 77–80% and 91–99%, respectively (Okamoto et al. 1992). HCV genetic diversity does not apply to the whole genome. Highly conserved regions are crucial for RNA replication and translation, as are the regions containing structural domains at each end of the HCV genome (the 5′ and 3′ untranslated regions [UTR]). However, the most conserved region is the 5′ NCR, with about 90% sequence identity among different strains (Bukh et al. 1992). In turn, the most variable region is the one coding structural glycoproteins E1 and E2, known as hypervariable regions 1 and 2, respectively. These parts of the HCV genome show a nucleotide similarity of below 50%. The main reason for genetic divergence among individual HCV isolates (quasispecies) is the high error rate of RNA-dependent RNA polymerase. HCV constantly mutates, and it is estimated that about 10^4 substitutions can occur in one cycle of replication. The genetic

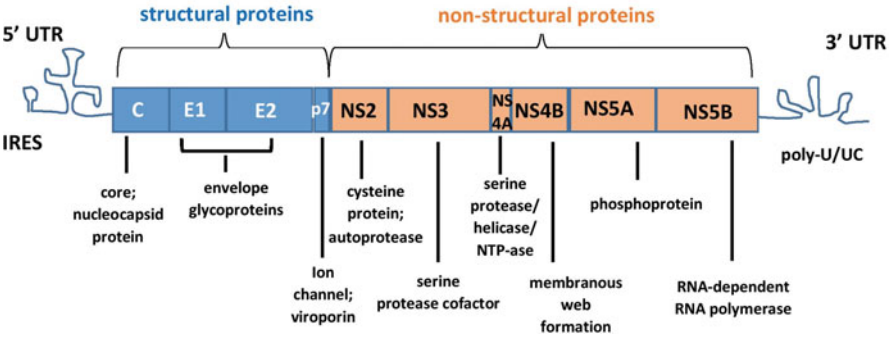

Fig. 1 Organization of the HCV genome and polyprotein cleavage products. The HCV genome contains a single open reading frame flanked by 5′ as well as 3′ noncoding regions. An internal ribosome entry site (*IRES*) is located within the 5′ part of the HCV genome and includes stem-loops that are necessary to start the translation of virus proteins. After translation, the HCV polyprotein is processed both by host and viral proteases into structural and nonstructural proteins (Friebe and Bartenschlager 2002); *UTR* untranslated region

Table 1 The distribution of HCV genotypes worldwide (Based on meta-analysis data from Messina et al. 2015)

HCV genotype	Regions of the most prevalence	Global prevalence of genotype [%]
1	Caribbean, Andean, Latin America, Central Europe, Southern Latin America, Central America, Asia, Central Latin America, Southeast Asia	46.2
2	Asia, Western sub-Saharan Africa	9.1
3	South Asia	30.1
4	Central sub-Saharan Africa, North and Middle East Africa	8.3
5	Southern sub-Saharan Africa	0.8
6	East Asia, Southeast Asia	5.4

heterogeneity of HCV has a crucial influence on its persistence (by evading the immune system) and the failure to develop a vaccine.

HCV genotypes and subtypes are distributed differently worldwide. The most globally prevalent are genotypes 1 (46.2%) and 3 (30.1%), while the least prevalent is genotype 5 (0.8%) (Table 1; Messina et al. 2015). HCV genotyping is very important for many clinical reasons. The most essential aspects of antiviral therapy (type and duration) can be optimized based on genotype information. Taking into account treatment with peg-IFN-α + RBV, genotypes 1 and 4 are considered "difficult to treat," whereas 2 and 3 are "easy to treat." These terms result from the fact that patients with genotype 2 or 3 are more likely to achieve sustained virologic response (SVR) (complete virus eradication) after completion of peg-IFN-α + RBV treatment. Estimated treatment duration with the use of peg-IFN-α + RBV for genotypes 1 and 4 is 24, 36, or 48 weeks (based on on-treatment HCV clearance); for genotypes 2 and 3, it is 24 weeks.

Epidemiological and Clinical Relevance

HCV infection is a growing health problem. As mentioned in the introduction, it is estimated that 130–150 million people worldwide are infected by this virus. There are approximately three million new cases annually, and 350–500,000 people die each year from hepatitis C-related liver disorders, such as cirrhosis and HCC (according to the World Health Organization 2014). HCV is a blood-borne virus, and there are a few commonly known routes of HCV transmission: intravenous injection and sharing injection equipment, medical equipment, and healthcare procedures (e.g., hemodialysis); mother-to-child vertical transmission; and sexual exposure (Zaltron et al. 2012). However, in one third of cases, determination of the source of infection is not possible.

HCV infection is rarely diagnosed in the early stages due to the fact that in most cases (~70–80%) it is asymptomatic. The disease is commonly diagnosed at the age of 30–50 in patients with intermediate or advanced pathological changes in the liver. The antibody to HCV can be detected within 1–3 months after exposure and HCV

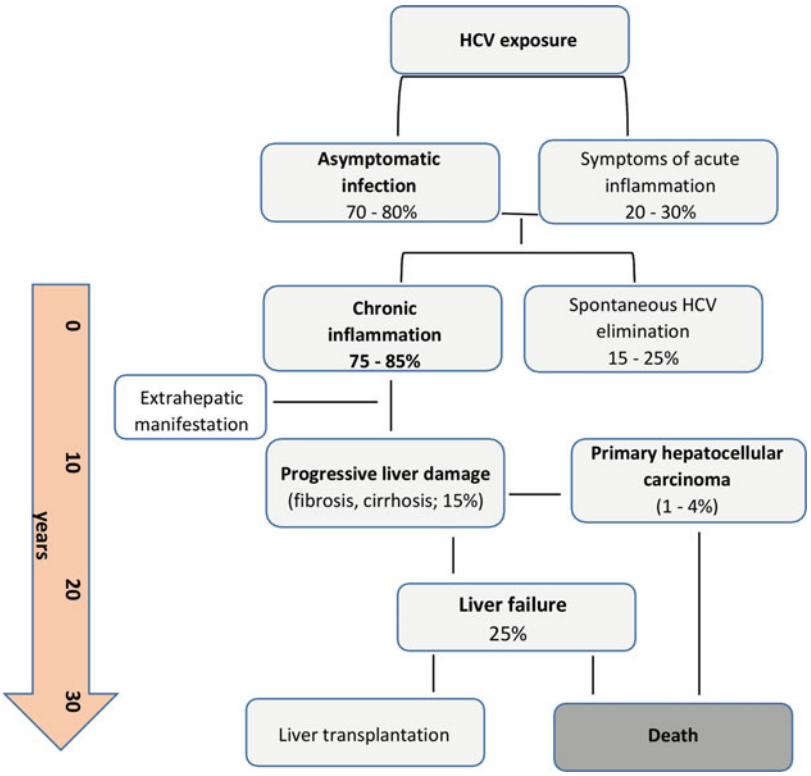

Fig. 2 Natural course of HCV infection. After HCV exposure, acute inflammation may develop, with nonspecific symptoms (20–30%) or asymptomatic infection (7–80%). In most cases (75–85%), inflammation becomes chronic and may be ongoing for many years without symptoms, leading to progressive liver damage (fibrosis and cirrhosis) and even hepatocellular carcinoma (1–4%). HCV infection is considered the leading cause of liver damage and the development of primary liver cancer (Chen and Morgan 2006)

RNA within 1–2 weeks. Only in about 15–25% of infected patients is spontaneous HCV clearance observed. The remaining patients (75–85%) develop chronic infection (Chen and Morgan 2006; Fig. 2).

CHC is defined by the persistence of HCV RNA in serum for at least 6 months from the beginning of the acute phase. CHC is considered as the leading cause of liver cirrhosis and HCC development (Alter 1997). CHC is known as the silent killer, on the grounds that progression of the pathological changes in the liver is very often asymptomatic, so that patients are not aware of being infected until they develop end-stage liver disorders or HCC. The time period from HCV infection to liver cirrhosis or HCC development depends on many factors, so cannot be predicted for all patients.

The main goal of antiviral therapy is HCV eradication, as well as the prevention of liver damage, HCC development, and death. There are currently only a few methods

of CHC treatment available. The first group of methods is based on interferon and includes dual therapy (peg-IFN-α + RBV) as well as triple therapy: peg-IFN-α + RBV combined with virus protease inhibitors, known as direct-acting antivirals (DAAs). Dual therapy can be used against all HCV genotypes, whereas triple therapy is only effective against genotype 1. DAAs include the first-generation inhibitors telaprevir and boceprevir and the second-generation simeprevir and sofosbuvir. The effectiveness of dual therapy ranges from 40% to 80% based on HCV genotype, while adding DAAs increases the cure rate up to about 70%. The second, quite new, group of methods is based on oral IFN-free drugs and includes combinations of sofosbuvir/simeprevir, sofosbuvir/daclatasvir, and sofosbuvir/RBV (depending on the HCV genotype) (EASL Recommendations on Treatment of Hepatitis C 2015). These drugs are very effective (80–95%) and allow the reduction of treatment duration to 12 or even 8 weeks. IFN is frequently poorly tolerated by patients and its administration results in a lot of, often serious, adverse effects so that many patients do not finish the treatment. The introduction of IFN-free drugs, therefore, arouses new hope in the treatment of CHC patients. The main disadvantage of IFN-free drugs, as referred to above, is their high price, which results in poor accessibility even in high-income countries.

microRNAs

Biogenesis and Function

miRNAs are a class of small (~22 nt), endogenous, noncoding RNAs. Genes encoding miRNAs are located either within the intronic region of protein-coding genes and the intronic or exonic regions of noncoding RNA or between independent transcription units. Intronic miRNA can be transcribed from a promoter of the host genes as well as from independent promoters, which allows independent control of the transcription process (Monteys et al. 2010). miRNA genes are often organized in clusters and are transcribed as multicistronic transcriptional units (Lagos-Quintana et al. 2001).

In general, the pathway that processes miRNA to a mature molecule consists of the following steps: transcription by polymerase RNA II or III (e.g., viral miRNAs), cleavage by Drosha/DGCR8, export to the cytoplasm, cleavage by Dicer, strand selection, and then loading into an RNA-induced silencing complex (RISC). In the nucleus the miRNA gene is transcribed to primary miRNA (pri-miRNA) of ~80 nt length with a 5′ cap and a 3′ poly-A tail. Then the pri-miRNA is processed by the microprocessor complex (Drosha/DGCR8) into a ~70 nt miRNA hairpin precursor (pre-miRNA) molecule. In addition to the aforementioned classical way, there is an alternative means of processing pre-miRNA – mitrons. Mitrons are alternative, hairpin precursors to miRNA. Their genes are located within introns and transcribed with the use of a messenger RNA (mRNA) splicing mechanism without microprocessor complex involvement. Next, the pre-miRNA is transported to the cytoplasm by an exportin-5 (Exp5) protein complex. There it is further processed by Dicer/TRBP/

PACT into a 21–24 nt miRNA/miRNA* duplex, composed of a guide and a passenger strand (labeled with an asterisk). On the basis of the stability of the 5′ end, one of the strands is often degraded, and the remaining one (a mature miRNA molecule) is loaded into the RISC complex, where it binds to an Argonaute (Ago) protein.

Within the RISC complex, the miRNA is protected from degradation and recognizes the target mRNA sequence. Recognition of target mRNA may occur with different complementarities. A perfect miRNA/mRNA match results in mRNA degradation, while an imperfect one in translation inhibition (Lewis et al. 2005). While mRNA transcript degradation leads to a decrease in the total amount of transcripts, the inhibition of translation does not change this amount because of the transport of transcripts to cytoplasmic P bodies (Liu et al. 2005). miRNA binding sites are usually located within the 3′ UTR of mRNA, less often within the 5′ UTR. Interestingly, binding within the 5′ UTR may result in an enhancement of mRNA stability and activation of its expression (Janowski et al. 2007). Interaction between miRNA and mRNA occurs through the conserved region present in positions 2–7 of the 5′ part of the miRNA sequence, called the "seed" region. Based on complementarity within the 5′ seed region of miRNA and the 3′ region of mRNA, miRNA-mRNA interactions can be divided into three main groups: canonical, 3-′-supplementary, and 3′-compensatory. The most abundant of the identified sites are canonical binding sites, which include 7mer-1A (with adenine opposite base 1 of the miRNA), 7mer-8m (with an additional pairing at position 8), and 8mer (with adenine opposite base 1 of the miRNA and with an additional pairing at position 8) sites. For highly conserved miRNA, 7mer sites are the most frequently observed (Lewis et al. 2005).

miRNAs play a crucial role in the posttranscriptional regulation of gene expression. Therefore, they regulate many important biological processes, such as cell proliferation, differentiation, apoptosis, endocytosis, and signaling, and are implicated in both physiological and pathological conditions. So far, approximately 2,600 human miRNAs have been identified (according to miRBase 21, 2014). One miRNA can regulate thousands of mRNAs, and one mRNA can be regulated by thousands of miRNAs (Lewis et al. 2005). It is estimated that about 60% of human genes are regulated by miRNAs (Cho 2012).

As Biomarkers of Diseases

Altered miRNA profiles have been reported in many human diseases. Deregulated miRNA expression occurs in a number of cancers, such as colon, breast, stomach, prostate, pancreas, and liver, where miRNAs may act as tumor oncogenes as well as suppressors and may control cancer cell proliferation, metastasis, and chemoresistance. Moreover, in carcinomas the miRNA profile is often tissue- and tumor specific (Volinia et al. 2006). miRNAs are also found to be implicated in various diseases: neurological, cardiovascular, autoimmune, infectious, muscular, diabetic, and many others. Whether altered miRNA expression results from disease or is the cause of disease is a matter of debate.

The specificity of the miRNA profile in various diseases has prompted researchers to run studies on their use as biomarkers. Currently, many miRNAs are shown as potential diagnostic, prognostic, and predictive biomarkers and even as therapeutic targets. It is believed that miRNAs are present in the circulation through three main mechanisms: cell death (apoptosis, necrosis), secretion in exosomes, and exocytosis (Kosaka et al. 2010). miRNAs may be released from various tissues and organs into the blood in both physiological and pathological conditions. It is significant that circulating cell-free miRNAs, in contrast with long RNAs, are resistant to RNase digestion. The ability to analyze miRNAs in body fluids makes them potentially useful candidates for biomarkers. It has been shown that the level of circulating miRNAs corresponds to pathological processes in tissues/organs and allows discrimination between patients and controls. Moreover, it has been found that the level of circulating miRNAs may correspond with disease severity, stage, outcome, and response to therapy.

Examples of miRNAs identified as potential biomarkers of diseases are shown in Table 2.

Regulation of the HCV Life Cycle

Growing evidence has documented that miRNAs are involved in the development and progression of HCV infection. They affect both host immune response to infection and HCV life cycle. Up to 52% of innate immune genes have conserved miRNA binding sites, which suggests the importance of these molecules in the regulation of immune response. In this subsection is discussed the impact of miRNAs on the HCV life cycle.

In general, the HCV life cycle consists of the following steps: entry to hepatocytes by receptor-mediated endocytosis, the uncoating of viral genomes, replication, translation and protein processing, and virion assembly and release (Fig. 3). There is evidence that miRNAs may regulate almost all of these steps (Li et al. 2014; Mekky et al. 2015; Sendi et al. 2015).

HCV entry to hepatocytes involves membrane receptors, such as glycosaminoglycans, low-density lipoprotein receptors, scavenger receptor B1, CD81, claudin-1, and occludin (Meredith et al. 2012). It has been found that miR-194 and miR-122 may hinder HCV entry to hepatocytes and decrease the amount of HCV RNA present by targeting CD81 and occludin, respectively (Mekky et al. 2015; Sendi et al. 2015). Moreover, it has been shown that miRNAs directly bind to the HCV genome and regulate HCV RNA replication and protein translation. Through binding to the 5′ and 3′ UTRs of the HCV genome, miR-122 enhances its replication and translation as well as stabilizing the genome by protecting it from exonuclease Xrn1. On the other hand, the binding of miR-196b, miR-199a, miR-448, and let-7b results in inhibition of HCV RNA replication (Li et al. 2014). The miRNAs that directly influence HCV life cycle are presented in Fig. 3.

Table 2 Circulating miRNAs as biomarkers of diseases

Condition	Body fluid	miRNA	Expression	AUC[b]	Reference
Alzheimer's disease	Cerebrospinal fluid	miR-125b	Down	Alzheimer's versus NINDCs[c]	Galimberti et al. (2014)
				0.82	
Triple-negative breast cancer (TNBC)	Plasma	miR-199a-5p	Down, correlation with TNM[a] stage and tumor subtypes	TNBC versus non-TNBC and healthy controls	Shin et al. (2015)
				0.88	
Acute graft-versus-host disease (aGVHD)	Plasma	miR-586	Up	aGVHD versus non-aGVHD	Wang et al. (2015b)
				0.74	
Nephroblastoma (Wilms tumor)	Serum	miR-100-5p	Up	Patients versus healthy controls	Ludwig et al. (2015)
		miR-130b-3p		0.94	
				0.90	
Osteosarcoma	Serum	miR-199a-5p	Up	Patients versus healthy controls	Zhou et al. (2015)
				0.86	
Hepatocellular carcinoma	Serum	miR-182	Up	Patients versus benign liver disease and healthy controls	Chen et al. (2015)
		miR-331-3p		0.91	
				0.89	
Bladder cancer	Urine	miR-214	Down, correlation with tumor stage and node status grade	Patients versus healthy controls	Wang et al. (2015a)
				0.84	

T tumor, *N* nodes, *M* metastasis
[a]TNM – classification of malignant tumors
[b]AUC – area under the receiver-operating characteristic curve
[c]NINDCs – noninflammatory neurological controls

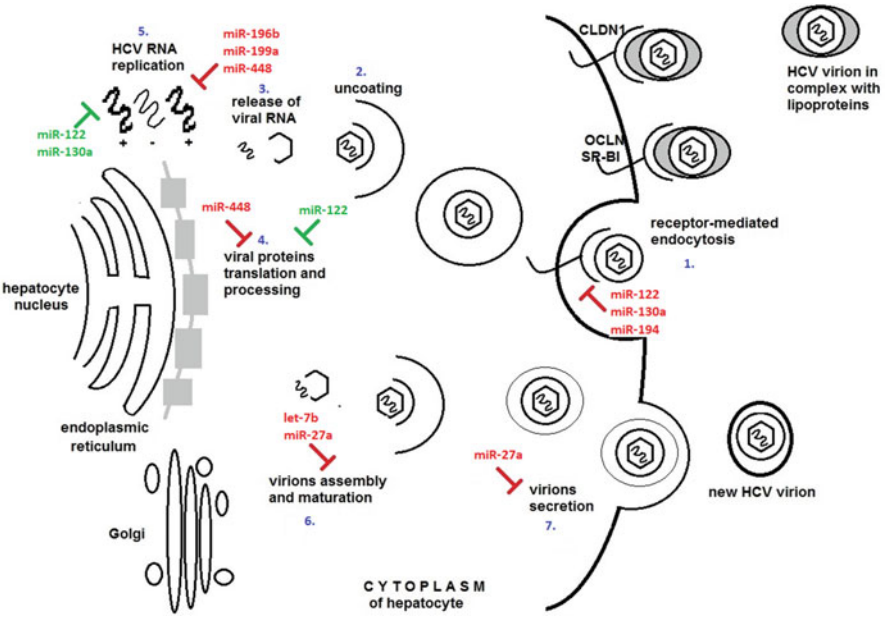

Fig. 3 microRNAs that directly and indirectly affect the HCV life cycle. In general, the HCV life cycle consists of the following steps: entry to hepatocytes by receptor-mediated endocytosis, uncoating of the viral genome, replication, translation and protein processing, and virion assembly and release (Li et al. 2014). miRNAs that have an inhibitory influence on particular steps in the HCV life cycle are marked in *red*, while those with a favorable impact are marked in *green* (Shirasaki et al. 2013; Li et al. 2014; Mekky et al. 2015; Sendi et al. 2015). *ApoB* and *ApoE* apolipoproteins B and E, *ER* endoplasmic reticulum

Regulation of Biological Pathways Related to the Course of HCV Infection

miRNAs, besides having a direct influence on HCV life cycle, may also affect HCV infectivity, understood as the number of complete, mature HCV particles capable of infecting naïve cells, as well as the development and course of HCV infection by regulating biological pathways. Analysis in silico reveals that miRNAs regulate up to 60% of human genes, including those that encode transcriptional and growth factors, cytokines, kinases, tumor suppressors, oncogenes, etc. These molecules also regulate HCV infection-related cellular pathways, such as IFN-mediated antiviral defense, IkB kinases (IKKs), mitogen-activated protein kinases (MAPKs), Janus kinase-signal transducer and activator of transcription (JAK-STAT), and lipid metabolism.

miRNAs may indirectly affect HCV virion assembly and release by regulation of lipid metabolism. It was reported that miR-27a decreased HCV infectivity, through the regulation of a number of lipid metabolism-related genes, crucial for viral particle production and formation (Shirasaki et al. 2013). Moreover, miRNAs may modulate IFN-induced antiviral defense through the control of IFN production, IFN

signaling, and the expression of IFN-stimulated genes (ISGs). IFNs are a group of signaling proteins that were first identified as molecules displaying antiviral activity (Isaacs and Lindenmann 1957). IFNs are produced in and released from host cells in response to the presence of viral nucleic acid as well as tumor cells. There are three classes of IFN: types I, II, and III. IFN type I comprises IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-k, and IFN-o; IFN type II is a single molecule – IFN-γ; IFN type III consists of IFN-λ1, IFN-λ2, and IFN-λ3. Categorization into the aforementioned classes is mainly based on the receptors with which these molecules interact. All three types of IFN play an essential role in antiviral defense and the modulation of immune response. It has been shown that expression of certain miRNAs can be up- or downregulated by all three classes of IFN, suggesting the important role of miRNAs in IFN-mediated response to infection (Sedger 2013).

Regulation of the immune system by miRNAs is crucial for the development and course of CHC, not only because of the regulation of antiviral response but also due to the development of pathological lesions in the liver. Although HCV itself is not cytopathic, HCV-related liver damage in the course of CHC is associated with immune-mediated mechanisms. miRNAs may modulate the action of immune cells, including T, B, and antigen-presenting cells, and the activation of stellate cells, which are involved in the expression of cytokines and growth factors and the production of nitric oxide. All these activities are important in the development of liver fibrosis and cirrhosis. Moreover, as mentioned earlier, miRNAs may regulate a number of genes that act as tumor suppressors or oncogenes and, therefore, control development of HCV-related HCC.

miRNAs that regulate IFN-dependent antiviral response, as well as the development of HCV-related liver damage, are summarized in Table 3.

miRNA-155 and miR-196b in Chronic Hepatitis C

This chapter will discuss the significance of two miRNAs – miR-155 and miR-196 – as potential diagnostic, prognostic, and predictive biomarkers in CHC. These molecules were selected as representative examples of the potential usefulness of miRNAs in monitoring HCV infection, CHC development, and the course of as well as response to antiviral treatment.

miRNA-155

The pri-miRNA-155 molecule is encoded within exon 3 of host gene *MIR155HG*, which is located on chromosome 21q21.3 and covers 13 kilobases (kb). *MIR155GH* was formerly called the B-cell integration cluster (BIC), on the grounds that it was identified in B-cell lymphomas as a proviral insertion site in proto-oncogenes (Clurman and Hayward 1989).

The pre-miR-155 molecule is devoid of an ORF, yet comprises a partial mismatched stem-loop, which is the most conserved part of the molecule.

Table 3 miRNAs that regulate IFN-mediated antiviral response and development of HCV-related liver damage

miRNA	Target genes	Function	Reference
miR-21	*MyD88*	Inhibition of IFN type I production	Chen et al. (2013)
	IRAK1		
miR-22	*IRF-5*	Reduction of IFN-stimulated gene expression	Polioudakis et al. (2013)
miR-145	*STAT1*, *STAT2*	Suppression of apoptosis	Gregersen et al. (2010)
miR-155	*IFN-γRα*	Inhibition of IFN-γ signaling	Banerjee et al. (2010)
miR-196b	*Bach1*	Increased hepatocyte resistance to oxidant injury, suppression of HCV RNA	Zhu et al. (2008)
miR-939	*iNOS*	Decreased nitric oxide production in hepatocytes	Guo et al. (2012)
miR-485-5p	*EMMPRIN*	Repression of HCC invasion and metastases	Sun et al. (2015)

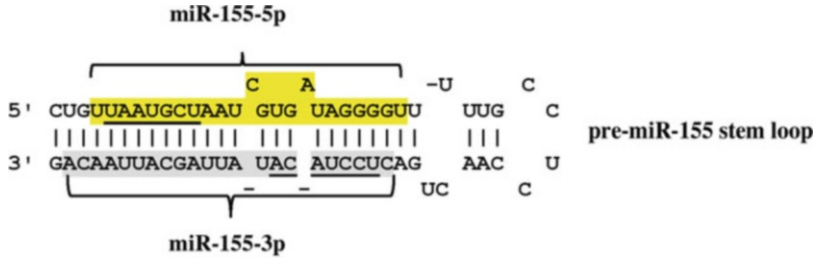

Fig. 4 The sequence of the pre-miRNA-155 stem-loop, the most conserved part of the molecule. Pre-miRNA-155 is cleaved and unfolded, forming the mature miR-155 molecule. Depending on which arm of the pre-miRNA-155 the mature miRNA-155 was created, miRNA-155-5p (most abundant, highlighted in *yellow*) or miRNA-155-3p (less abundant, highlighted in *gray*) is formed. The "seed" sequence required for interaction with the target mRNA is *underlined* (miRBase 21, 2014)

Pre-miR-155 of approximately 70 nt in length is processed into 22 nt mature molecules, known as miR-155-5p or miR-155-3p, depending on which arm of the pre-miR the mature miRNA was created (Fig. 4).

Expression and Function

It has been found that miRNA-155 plays a crucial role in regulating immune response mechanisms. It is expressed in many immune cells, such as T and B lymphocytes, macrophages, granulocytes, and dendritic cells. Expression of miRNA-155 is induced during macrophage inflammatory response by a number of inflammatory mediators, including IFN-β, IFN-γ, bacterial lipopolysaccharides (LPSs), polyriboinosinic-polyribocytidilic acid (poly-I:C), and tumor necrosis

factors (TNFs) α and β. Induction of the miR-155 transcription signal is mediated by the c-Jun N-terminal kinase (JNK) pathway and may be controlled by the MAPK pathway (O'Connell et al. 2007). Multiple studies have revealed that miRNA-155 is crucial for proper immune function, as well as generating pro-inflammatory states and enhancing immune response. Studies on miRNA-155-deficient mice have demonstrated meaningful disturbances in B- and T-cell function and the production of IFN-γ and interleukin-2 (Rodriguez et al. 2007). It was shown that miRNA-155 regulated differentiation of lymphocytes T CD4+ and CD8+, as well as B lymphocyte and antibody production. Expression of miRNA-155 in CD8+ T cells influences their effector and memory responses. The way in which miRNA-155 takes part in the development of memory responses is still unknown, but is a subject of interest, especially in the context of chronic infections and vaccine development (Seddiki et al. 2013). miRNA-155 is downregulated by interleukin-10 (IL-10), which is the main anti-inflammatory cytokine, whereas inhibition of miRNA-155 results in decreased expression of TNF-α and interleukin-6 (pro-inflammatory cytokines) and increased expression of IL-10 (Billeter et al. 2014).

miRNA-155 is considered a multifunctional miRNA molecule. Like other miRNAs, it has hundreds of putative target mRNAs (the validated miRNA-155 target genes selected are shown in Table 4). It has been demonstrated that miRNA-155 plays an important role, not only in immune response but also in various physiological (e.g., hematopoiesis) as well as pathological processes, including a wide range of diseases: cancerous, neurological, neoplastic, cardiovascular, autoimmune, etc. miRNA-155 is expressed in lymphoid and myeloid cells and regulates human myelopoiesis and erythropoiesis (Masaki et al. 2007). In cancerogenesis, miRNA-155 seems to be a link between inflammation and cancer. Altered miRNA-155 expression has been found in Hodgkin's, Burkitt's, and diffuse large B-cell lymphomas and in breast, cervical, colon, and lung cancers. It appears that miRNA-155 oncogenicity may be associated with its role in the regulation of apoptosis (Faraoni et al. 2009).

Prediction and Prognosis of Chronic Hepatitis C

As mentioned, miRNA-155 is a crucial regulator of immunity, acting as a pro-inflammatory factor. Studies on miRNA-155 have shown its involvement in both viral and bacterial infectious diseases, such as human T-cell leukemia virus (HTLV), Epstein-Barr virus (EBV), Borna disease virus (BVD), and reticuloendotheliosis virus strain T (REV-T), and with *Mycobacterium tuberculosis*, *Helicobacter pylori* and *Listeria monocytogenes* (Zeng et al. 2015). Based on miRNA-155 involvement in immune response, the interest in miRNA-155 in the context of HCV infection is justified by its potential influence on HCV elimination, the development of chronic inflammation, and the progress of liver damage.

Expression of miRNA-155 was found to be upregulated in the sera, peripheral blood mononuclear cells (PBMCs), and hepatocytes of CHC patients in comparison with healthy subjects. It has been suggested that this molecule contributes to generating a pro-inflammatory state and may have an impact on the course of

Table 4 Selected, experimentally validated targets for miRNA-155 (miRWalk 2.0, June 2015)

Gene	Full name[a]
BACH1	BTB and CNC homology 1 and basic leucine zipper transcription factor 1
BCL6	B-cell CLL/lymphoma 6
CD81	CD81 molecule
CLDN1	Claudin-1
EGFR	Epidermal growth factor receptor
IFNG1	Interferon-gamma receptor 1
IL6	Interleukin 6
IRAK3	Interleukin-1 receptor-associated kinase 3
MAP3K10	Mitogen-activated protein kinase kinase kinase 10
MEIS1	Meis homeobox 1
MYD88	Myeloid differentiation primary response 88
SMAD	SMAD family member 1
STAT3	Signal transducer and activator of transcription 3
TP53INP1	Tumor protein p53 inducible nuclear protein 1

[a]Acquired from the National Center for Biotechnology Information, NCBI

HCV infection and the development and progression of chronic inflammation (Bala et al. 2012; Zhang et al. 2012). It is believed that the mechanism responsible for miRNA-155 induction is a constitutively active NF-kB pathway. It has been shown that HCV core and nonstructural proteins (NS3 and NS5) or toll-like receptors (TLRs) 4 and 8 can mediate miRNA-155 upregulation. This TLR-mediated signal transduction is dependent on IKKγ/NEMO, which is a component of the NF-kB pathway (Rai et al. 2008).

Interestingly, it was also demonstrated that BIC, the precursor to mature miRNA-155, may play a role in CHC. Both mature miRNA-155 and BIC are transcribed from the same primary molecule, pri-miRNA-155. Rai et al. (2008) found a precise correlation between miRNA-155 and BIC expression in the diffuse large B-cell lymphoma cell line. It is supposed that miRNA-155 may be released from BIC transcripts, spliced and polyadenylated RNA BIC, as well as non-spliced RNA BIC. While expression of the BIC spliced form was often higher than the level of non-sliced, the amount of spliced cytosolic BIC closely reflected the amount of non-spliced transcripts. These results suggest that analysis of BIC may be a surrogate measure for mature miR-155 (Rai et al. 2008). Sidorkiewicz et al. (2010) revealed that BIC is upregulated in the PBMCs of CHC patients. Moreover, they analyzed the expression of BIC before and after antiviral treatment and showed an association between BIC expression and the presence of HCV RNA in serum and PBMCs. Patients who had completely eliminated HCV RNA had a lower BIC expression level. However, patients with HCV RNA in their sera and PBMCs had a higher BIC expression in their PBMCs. These results suggest the existence of a correlation between BIC and HCV presence and replication. The authors speculated that BIC and miRNA-155 may modulate the function of PBMCs promoting HCV persistence and may, therefore, influence the effectiveness of IFN-α-based therapy (Sidorkiewicz et al. 2010). The link between BIC/miRNA-155 and HCV RNA

replication was further confirmed by the same team in the PBMCs of CHC patients (Grek et al. 2011). Interestingly, Grek et al. demonstrated that BIC RNA shows an upward tendency when an antigenomic HCV RNA strand is detected in PBMCs, independently of the presence of HCV RNA in sera. These results indicate that in the case of a lack of HCV RNA in the serum, increased BIC level may suggest HCV persistence in PBMCs and the possibility of the recurrence of HCV RNA replication. Therefore, measurement of the BIC RNA level could be useful in supplementing the measurement of HCV RNA in sera, in order to evaluate the effectiveness of antiviral treatment.

It appears that miRNA-155 expression differs depending on the material analyzed (e.g., serum, PBMCs, or liver tissue) and HCV genotype. In patients infected with HCV genotypes 1, 2, and 3, upregulated miRNA-155 expression was found in their sera, PBMCs, and liver when compared to healthy controls (Bala et al. 2012; Zhang et al. 2012). By contrast, no significant difference in any of the three aforementioned materials was observed in patients with HCV genotype 4 when compared to healthy controls. However, Riad et al. (2015) investigated the usefulness of the analysis of serum miRNA-155 expression level as a noninvasive prognostic and predictive biomarker in patients infected with HCV genotype 4. Studies revealed that patients with SVR had significantly higher pretreatment levels of miRNA-155 compared with patients who did not respond to treatment (NR). The authors suggest that miRNA-155 may be a positive predictor of response to treatment with peg-IFN. Moreover, miRNA expression in the sera was positively correlated with alanine and aspartate aminotransferases (ALT and AST, respectively), which also renders miRNA-155 as a promising prognostic marker of CHC infection. The authors remark, however, that these results are not in line with those of studies on HCV 1, 2, and 3 genotype patients, where miRNA-155 was found to be downregulated in the sera and PBMCs of SVR patients after antiviral therapy (Riad et al. 2015). It was also recently found that miRNA-155 was downregulated in natural killer (NK) lymphocytes from CHC patients (Yong et al. 2015). In turn, the reconstitution of miRNA-155 in these cells resulted in enhancement of IFN-γ production. Regulation of IFN-γ production with the participation of miRNA-155 occurs through inhibiting T-bet and downstream Tim-3 expression. T-bet is a transcription factor for Tim-3 in T helper type I lymphocytes and is responsible for controlling NK cell maturation. The authors suggest that miRNA-155 may regulate Tim-3/T-bet/STAT5 signaling and cytokine suppression in NK cells and thereby maintain balance between immune clearance and injury in the course of CHC (Yong et al. 2015). Jiang et al. (2014) found increased miRNA-155 expression in the hepatocytes of CHC patients and an adverse correlation between the expression of miRNA-155 and IL-10 and TNF-β. The authors demonstrated that hepatic miRNA-155 level was negatively correlated with ISG expression. Moreover, patients with SVR showed a higher serum and hepatic miRNA-155 expression in comparison with NR subjects to peg-IFN-α + RBV antiviral treatment. Elevated serum miRNA-155 expression was also associated with genotype CC of IL28B, which is known as a molecular predictor of HCV clearance. Therefore, it was concluded that a higher expression

of miRNA-155 was beneficial for patients and may be a positive prediction factor in the treatment of CHC with peg-IFN-α + RBV.

As mentioned earlier, the regulatory function of miRNA-155 is considered to be a factor linking inflammation and cancerogenesis. In regulating immune response cells, miRNA-155 has an influence on the development and course of chronic inflammation. It is widely recognized that chronic inflammation in the liver leads to progressive tissue damage. In the course of chronic inflammation, activated cells secrete a number of cytokines and growth factors, which results in an imbalance between the production and degradation of the extracellular matrix (ECM) and in a progressive accumulation of its components. Replacement of damaged hepatic parenchyma by elements of connective tissue (scar tissue) blocks the portal flow of blood through the organ, thereby disturbing its function. Moreover, matrix degradation and the accumulation of ECM components lead to changes in the structure of the liver and to the development of cirrhosis. Therefore, it seems that analysis of miRNA-155 expression may also be useful in the prognosis of the course of CHC and the development of fibrosis, cirrhosis, and HCC. It is worth mentioning that CHC is considered a major cause of liver tissue damage and the development of primary HCC, and HCV-related cirrhosis has become the most common indication for liver transplantation.

miRNA-155 is considered to be an oncogenic miRNA, and its expression has been found to be upregulated in many cancers, including HCC. In colorectal cancer, miRNA-155 overexpression promotes the migration and invasion of cancer cells and correlates with poor prognosis. The mechanism by which miRNA-155 regulates cell metastasis is by targeting claudin-1 expression (Zhang et al. 2013). It was also demonstrated that the oncogenic properties of miRNA-155 result from regulating suppressor of cytokine signaling 1 and mediating epithelial-to-mesenchymal transition. In HCC, increased miRNA-155 expression results in the promotion of cancer cell proliferation through inhibition of the expression of CCAAT/enhancer-binding protein beta (C/EBPβ) (Zhang et al. 2012). Zhang et al. found that there is a pathway mediated by NF-kB/miRNA-155 that promotes formation of HCC. The authors postulate that NF-kB/miRNA-155 is a crucial component of the pathway controlling development of HCV-related HCC. Increased miRNA-155 expression leads to activation of HCC cell proliferation and inhibition of apoptosis through Wnt/β-catenin signaling, both in vitro and in vivo. In turn, inhibition of miRNA-155 results in stopping cells at the G0/G1 cell cycle checkpoint. Moreover, one of the tumor suppressor genes – (*APC*), known to be a negative regulator of Wnt/β-catenin – is a direct target of miRNA-155 (Zhang et al. 2012).

It is also worth mentioning that upregulated miRNA-155 expression may be a promising prospective prognostic biomarker after liver transplantation. Liver transplantation (LT) is an effective treatment for various end-stage liver diseases. However, it is very often accompanied by complications responsible for graft loss, such as acute cellular rejection, recurrence of hepatitis C or HCC. Therefore, there is a need to establish biomarkers that allow better control of liver allograft viability. Han et al. (2012) revealed that patients with higher miRNA-155 expression in liver tissue after orthotropic LT had an elevated risk of HCC recurrence and shorter survival.

Asaoka et al. (2014) showed that acute cellular rejection can be diagnosed by measuring miRNA-122 and miRNA-155 expression in liver grafts. Moreover, expression of these molecules may be used as a biomarker that allows distinction between acute cellular rejection and the recurrence of hepatitis C.

miRNA-196b

The miRNA-196 family contains three molecules: miRNA-196a-1, miRNA-196a-2, and miRNA-196b. Genes encoding these molecules are located in the regions of homeobox (HOX) clusters. *HOX* genes, also known as homeotic genes, are a group of 39 related genes, organized into 13 paralogous subgroups, which contain a homeobox domain and encode transcription factors essential for embryogenesis. Mammals have four HOX clusters, labeled from HOXA to HOXD. The miRNA-196a-1 gene is located on chromosome 17q21.32, between HOX9B and HOX10B, miRNA-196a-2 on chromosome 12q13.13 between HOX10C and HOX9C, and miRNA-196b on chromosome 7p.15.2 between HOX9A and HOX10A (Popovic et al. 2009). The miRNA-196a-1 and miRNA-196a-2 genes transcribe the same mature miRNA, whereas the miRNA-196b gene transcribes a molecule that differs from miRNA-196a by a single nucleotide. The pri-miRNA-196b stem-loop and mature miRNA-196b sequence are shown in Fig. 5.

Prediction and Prognosis of Chronic Hepatitis C

The miRNA-196 family plays important roles in physiological as well as pathological development through targeting several transcription factors and regulatory molecules, such as HOXB8, HOXC8, annexin A1 (ANXA1), and nuclear architectural factor (HMAGA2) (Table 5).

It has been reported that miR-196b expression is significantly induced by IFN-β in the human hepatoma cell line. This observation suggests that miRNA-196 may be a component of the host antiviral mechanism and play an important role in immune response modulation. Moreover, it was revealed that miRNA-196b directly interacts with the HCV genome. miRNA-196b binds to the region coding NS5 and disturbs the HCV life cycle by RNA replication inhibition (Pedersen et al. 2007; Li et al. 2014). Hou et al. (2010) demonstrated that miRNA-196b represses BTB and CNC homology 1 and basic leucine zipper transcription factor 1 (BACH1), upregulates heme oxygenase 1 (HMOX1), and thereby inhibits HCV RNA replication and protein translation in vitro. The authors suggest that miRNA-196b may be the basis of a promising novel strategy to prevent HCV infection as well as HCV-related liver disease development.

Grek et al. (2011) examined miRNA-196b expression in correlation with the detection of an antigenomic HCV RNA strand in PBMCs. They revealed significantly higher expression of this molecule in the case of HCV RNA replication in PBMCs. These results are not in line with earlier in vitro studies. However, the possibility cannot be excluded that increased miRNA-196b expression is some kind of host defense mechanism in response to HCV infection. It has also been shown that

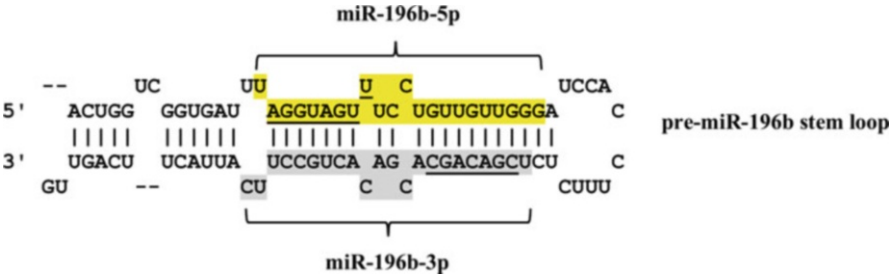

Fig. 5 The sequence of the pre-miRNA-196 stem-loop, the most conserved part of the molecule. Pre-miRNA-196b is cleaved and unfolded, forming the mature miRNA-196b molecule. Depending on which arm of the pre-miR-196b the mature miR-196b was created, miRNA-196b-5p (most abundant, highlighted in *yellow*) or miRNA-196b-3p (less abundant, highlighted in *gray*) is formed. The "seed" sequence required for interaction with the target mRNA is *underlined* (miRBase 21, 2014)

Table 5 Selected, experimentally validated targets for miRNA-196b (miRWalk 2.0, June 2015)

Gene	Full name[a]
ATF4	Activating transcription factor 4
BACH1	BTB and CNC homology 1 and basic leucine zipper transcription factor 1
BCL2	B-cell CLL/lymphoma 2
BUB1	BUB1 mitotic checkpoint serine/threonine kinase
CD8A	CD8a molecule
FAS	Fas cell surface death receptor
HOXB8	Homeobox B8
HOXC8	Homeobox C8
IARS	Isoleucyl-tRNA synthetase
MAT2A	Methionine adenosyltransferase II, alpha
MEIS1	Meis homeobox 1
TLE3	Transducin-like enhancer of split 3

[a]Acquired from the National Center for Biotechnology Information, NCBI

miRNA-196a may be a promising and sensitive candidate as a diagnostic biomarker in CHC. Liu et al. (2015) reported that patients with CHC had significantly downregulated serum miRNA-196a compared with healthy controls.

The molecular mechanism by which HCV leads to liver damage is still poorly understood. Some studies have revealed that this virus can directly induce oxidative stress in hepatocytes, which results in progressive liver injury. The generation of reactive oxygen species (ROS) is a very important mechanism for the course of CHC and the development of HCV-related liver diseases. Hou et al. (2010) showed that in vitro miRNA-196b targets *BACH1*, which is a transcriptional repressor of *HMOX1*. In turn, HMOX1 is a crucial cytoprotective enzyme, responsible for catalyzing heme degradation and generating molecules of anti-oxidative and anti-inflammatory activities. It has also been shown that HMOX1 suppresses HCV replication (Zhu et al. 2008). Therefore, miRNA-196b

indirectly, through repression of *BACH1*, upregulates *HMOX1* expression and increases hepatocyte resistance to oxidant injury. Taking into account the results of the aforementioned study, it seems that miRNA-196b may have a significant impact on the course of CHC and the development of HCV-related liver diseases (Hou et al. 2010).

The significance of the miRNA-196 family has been studied in several malignancies, such as breast, colorectal, esophageal, pancreatic, head, and neck cancers, and in a few types of leukemia (Chen et al. 2011). The mechanism of action of miRNA-196b in cancerogenesis is not clear and remains largely unknown. This molecule can have both oncogenic and tumor-suppressive properties. This impact on tumor development depends on the expression of its target mRNA. In the case that miRNA-196b targets oncogenes, it has suppressive properties, whereas when it targets tumor suppressors, it has mainly oncogenic characteristics. It is widely recognized that the properties of a single gene may differ between tissues – it can function as an oncogene in one type of tissue and as a tumor suppressor in others. However, it has been shown that miRNA-196b can simultaneously or sequentially repress oncogenic and tumor suppressor genes in the same tissue. Li et al. (2012) demonstrated that miRNA-196b targets both HOXA9/Meis homeobox 1 (*MEIS1*) oncogene and the Fas cell surface death receptor (*FAS*) tumor suppressor in rearranged mixed lineage leukemia (MLL). Nevertheless, the authors state that the main role of miRNA-196b in this type of cancer is the repression of crucial tumor suppressors and that higher miRNA-196b expression is associated with more aggressive leukemic phenotypes and unfavorable prognosis (Li et al. 2012). Similarly, Cao et al. (2015) reported that miRNA-196b inhibits apoptosis and suppresses differentiation in mouse marrow progenitor cells. They state that overexpression of miRNA-196b may play a crucial role in the development of rearranged MLL. In contrast, Rebucci et al. (2015) revealed that miRNA-196b inhibits cell proliferation and induces apoptosis in the hepatoma cell line through targeting insulin-like growth factor 2 RNA-binding protein 1 (*IGF2BP1*). Moreover, this molecule is downregulated in cells incubated under hypoxia; as is well understood, hypoxia induces chemoresistance and protects cancer cells from death. The authors suggest that miRNA-196b may regulate the chemoresistance induced by hypoxia. Therefore, it seems that in this case miRNA-196b can act as a component of tumor-preventing machinery. It has also been shown that all members of the miRNA-196 family may be potential suppressors of metastasis in vivo. Li et al. (2010) observed a precise correlation between the ratio of miRNA-196 to HOXC8 and metastasis status in breast cancer samples. They state, therefore, that the ratio of miRNA-196 to HOXC8 expression may be a potential biomarker of breast cancer metastasis.

On the basis of the abovementioned studies, it can be concluded that the role of the miRNA-196 family in tumor development and metastasis is not general and depends on the type of tissue from which the tumor originated and the balance between miRNA-196b expression and that of its target molecules, including oncogenes and tumor suppressors.

Potential Applications for the Prognosis of Chronic Hepatitis C, Other Diseases, or Conditions

This chapter has summarized recent studies on the role of two selected miRNAs – miRNA-155 and miRNA-196b – in HCV infection. It has discussed the significance and involvement of these molecules in regulating the HCV life cycle, the development of HCV infection and HCV-related liver diseases, as well as their influence on the course of CHC. Special emphasis has been given to their potential application as diagnostic, prognostic, and predictive biomarkers and as targets of novel antiviral therapies.

On the basis of the results of the various reports mentioned, it seems that miRNA-155 and its functional precursor BIC could potentially be useful in the diagnosis of HCV infection, as well as in the prognosis of the course and effectiveness of peg-IFN-α + RBV-based treatment. Upregulated expression of BIC in PBMCs may promote HCV replication in these cells. Moreover, after patients have completed antiviral treatment, it could be a determinant of HCV persistence and HCV replication recurrence. On the other hand, the overexpression of miRNA-155 in serum as well as liver tissue may be beneficial for CHC patients and was shown to be associated with HCV clearance and SVR. Pro-inflammatory activity of miRNA-155 and the regulation of the T lymphocyte response promote development of HCV-related liver injury, including fibrosis and cirrhosis. It was also evidenced that miRNA-155 has oncogenic properties and promotes liver tumor growth through enhancing cell proliferation and inhibiting apoptosis. Therefore, it seems that this molecule may be a marker of poor prognosis in patients with CHC. Similarly, overexpressed miRNA-155 expression in liver grafts could be useful as a marker of poor prognosis after liver transplantation.

The expression as well as function of miRNA-155 was examined in other diseases and conditions. It was shown that miRNA-155 plays a crucial role in myelopoiesis, erythropoiesis, and erythrocyte maturation. In rheumatoid arthritis (RA), expression of miRNA-155 is upregulated and may be useful in distinguishing RA from osteoarthritis. It was also shown that miRNA-155 may be involved in blood pressure regulation and its overexpression may be related with the development of hypertension and cardiovascular diseases. In adult non-Hodgkin's lymphoma, elevated miRNA-155 expression may determine poor prognosis, due to the fact that its expression is higher in the germinal center B-cell-like subtype than in the activated B-cell-like subtype. O'Connell et al. (2007) found increased miRNA-155 expression in patients with the M4 or M5 subtype of acute myeloid leukemia. Recent studies have revealed that miRNA-155 is also overexpressed in chronic lymphocytic leukemia (Faraoni et al. 2009). As mentioned, miRNA-155 plays an oncogenic role in several malignancies, such as breast, colon, lung, and pancreatic cancer, and its overexpression is generally associated with poor prognosis (Seddiki et al. 2013). Osaka et al. (2015) demonstrated that miRNA-155 may serve as a prognostic marker and potential therapeutic target in chordoma.

miRNA-196b was shown to have the opposite effect to miRNA-155. miRNA-196b binds directly to the HCV genome and inhibits HCV RNA replication.

Moreover, it was shown that overexpression of miRNA-196b may increase hepatocyte resistance to oxidant injury during CHC. The role of this molecule in cancerogenesis is complex, due to the fact that it can have both oncogenic and tumor-suppressive properties. Nevertheless, it was shown that miRNA-196b inhibits cell proliferation and induces apoptosis in human hepatoma cells. Therefore, it seems that miRNA-196b may be a promising, positive prognostic and predictive biomarker in patients with CHC.

The significance of the miRNA-196 family has been established in many malignancies, such as breast, colorectal, esophageal, pancreatic, head, and neck cancers, and in a few types of leukemia (Chen et al. 2011). It was shown that upregulated miRNA-196a expression is a promising candidate as a biomarker of the progression of Barrett's metaplasia to esophageal adenocarcinoma (Maru et al. 2009). Guan et al. (2010) demonstrated that miRNA-196 may be a useful marker of poorer survival and malignant progression in gliomas. Li et al. (2010) revealed that the expression ratio of miRNA-196 to HOXC8 is useful in establishing metastasis status in breast cancer. The miRNA-196 family is, consequently, considered to be a novel therapeutic target in many cancers (Chen et al. 2011).

Certainly, more research on the potential application of miRNA-155 and miRNA-196b as biomarkers must be conducted to draw firm conclusions. Nevertheless, the results of previously conducted studies allow us to recognize these molecules as potentially useful biomarkers in the prognosis and prediction of CHC and as promising targets of novel antiviral therapies, which prompts further research.

Summary Points

- This chapter focuses on the potential role of microRNAs (miRNAs), in particular miRNA-155 and miRNA-196b, as diagnostic, prognostic, and predictive biomarkers of hepatitis C virus (HCV) infection.
- miRNAs directly and indirectly regulate the HCV life cycle, as well as affecting the course of chronic hepatitis C (CHC) and the development of HCV-related liver diseases.
- CHC is a growing epidemiological problem, and there is a major need to identify biomarkers for monitoring the prognosis as well as prediction of treatment efficacy in patients with CHC.
- miRNA-155 is a crucial regulator of immune response of pro-inflammatory activities, which is considered as a factor linking inflammation and cancerogenesis.
- miRNA-155 may modulate the function of peripheral blood mononuclear cells (PBMCs), promoting HCV persistence and replication in these cells.
- Hepatic expression of miRNA-155 correlates with the serum level of biochemical markers of liver damage.
- miRNA-155 has oncogenic properties, promotes hepatocyte proliferation, and inhibits apoptosis.

- Higher miRNA-155 expression in liver tissue after liver transplantation may be an adverse prognostic factor associated with an elevated risk of hepatocellular carcinoma (HCC) recurrence and shorter survival.
- miRNA-196b directly interacts with a region of the HCV genome and disturbs HCV RNA replication and protein translation.
- Overexpression of miRNA-196b increases hepatocyte resistance to oxidant injury, inhibits cell proliferation, and induces apoptosis in the hepatoma cell line.

Acknowledgements This work was supported by National Science Centre Poland, grant no.: 2015/19/N/NZ6/02830.

References

Alter MJ. The epidemiology of acute and chronic hepatitis C. Clin Liver Dis. 1997;1:197–203.

Asaoka T, Hernandez D, Tryphonopoulos P, et al. Clinical significance of intragraft miR-122 and miR-155 expression after liver transplantation. Hepatol Res. 2014. doi:10.1111/hepr.12424 [Epub ahead of print].

Bala S, Tilhaun Y, Taha O, et al. Increased microRNA-155 expression in the serum and peripheral monocytes in chronic HCV infection. J Transl Med. 2012;10:151–61.

Banerjee A, Schambach D, DeJong CS, et al. MicroRNA-155 inhibits IFN-gamma signaling in CD4+ T cells. Eur J Immunol. 2010;40:225–31.

Bartenschlager R, Penin F, Lohmann V, et al. Assembly of infectious hepatitis C virus particles. Trends Microbiol. 2011;19:95–103.

Billeter AT, Hellmann J, Roberts H, et al. MicroRNA-155 potentiates the inflammatory response in hypothermia by suppressing IL-10 production. FASEB J. 2014;28:5322–36.

Bukh J, Purcell RH, Miller RH. Sequence analysis of the 5′ noncoding region of hepatitis C virus. Proc Natl Acad Sci U S A. 1992;89:4942–6.

Cao D, Hu L, Lei D, et al. MicroRNA-196b promotes cell proliferation and suppress cell differentiation in vitro. Biochem Biophys Res Commun. 2015;457:1–6.

Chen SL, Morgan TR. The natural history of hepatitis C virus (HCV) infection. Int J Med Sci. 2006;3:47–52.

Chen C, Zhang Y, Zhang L, et al. MicroRNA-196: critical roles and clinical applications in development and cancer. J Cell Mol Med. 2011;15:14–23.

Chen Y, Chen J, Wang H, et al. HCV-induced miR-21 contributes to evasion of host immune system by targeting MyD88 and IRAK1. PLoS Pathog. 2013;9:e1003248.

Chen L, Chu F, Cao Y, et al. Serum miR-182 and miR-331-3p as diagnostic and prognostic markers in patients with hepatocellular carcinoma. Tumour Biol. 2015. doi:10.1007/s13277-015-3430-2 [Epub ahead of print].

Cho W. MicroRNAs as therapeutic targets and their potential applications in cancer therapy. Expert Opin Ther Targets. 2012;16:747–59.

Choo QL, Kuo G, Weiner AJ, et al. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science. 1989;244:359–62.

Choo QL, Richman KH, Han JH, et al. Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci U S A. 1991;88:2451–5.

Clurman BE, Hayward WS. Multi proto-oncogene activations in avian leucosis virus-induced lymphomas: evidence for stage-specific events. Mol Cell Biol. 1989;9:2657–64.

EASL. Recommendations on treatment of hepatitis C 2015. J Hepatol. 2015;63:199–236.

Faraoni I, Antonetti FR, Cardone J, et al. MiR-155 gene: a typical multifunctional microRNA. Biochim Biophys Acta. 2009;1792:497–505.

Friebe P, Bartenschlager R. Genetic analysis of sequences in the 3′ nontranslated region of hepatitis C virus that are important for RNA replication. J Virol. 2002;76:5326–38.
Galimberti D, Villa C, Fenoglio C, et al. Circulating miRNAs as potential biomarkers in Alzheimer's disease. J Alzheimers Dis. 2014;42:1261–7.
Gregersen LH, Jacobsen AB, Frankel LB, et al. MicroRNA-145 targets YES and STAT1 in colon cancer cells. PLoS One. 2010 Jan 21;5(1):e8836. doi: 10.1371/journal.pone.0008836.
Grek M, Piekarska A, Bartkowiak J, et al. Coordinated increase of miRNA-155 and miRNA196b expression correlates with detection of the antigenomic strand of hepatitis C virus in peripheral blood mononuclear cells. Int J Mol Med. 2011;28:875–80.
Guan Y, Mizoguchi M, Yoshimoto K, et al. MiRNA-196 is upregulated in glioblastoma but not in anaplastic astrocytoma and has prognostic significance. Clin Cancer Res. 2010;16(16):4289–97.
Guo Z, Shao L, Zheng L, et al. miRNA-939 regulates human inducible nitric oxide synthase posttranscriptional gene expression in human hepatocytes. Proc Natl Acad Sci U S A. 2012;109:5826–31.
Han Z-B, Chen H-Y, Fam J-W, et al. Up-regulation of microRNA-155 promotes cancer cell invasion and predicts poor survival of hepatocellular carcinoma following liver transplantation. J Cancer Res Clin Oncol. 2012;138:153–61.
Hou W, Tian Q, Zheng J, et al. MicroRNA-196b represses Bach1 protein and HCV gene expression in human hepatoma cells expressing hepatitis C virus proteins. Hepatology. 2010;51:1494–504.
Isaac A, Lindenmann J. Virus interference, I. The interferon. Proc R Soc Lond Ser B: Biol Sci. 1957;147:258–67.
Janowski BA, Younger ST, Hardy DB, et al. Activating gene expression in mammalian cells with promoter-targeted duplex RNAs. Nat Chem Biol. 2007;3:166–73.
Jiang M, Broering R, Trippler M, et al. MicroRNA-155 controls Toll-like receptor 3- and hepatitis virus-induced immune responses in the liver. J Viral Hepat. 2014;21:99–110.
Kosaka N, Iguchi H, Yoshioka Y, et al. Secretory mechanism and intracellular transfer of microRNA in living cells. J Biol Chem. 2010;285:17442–52.
Lagos-Quintana M, Rauhut R, Lendeckel W, et al. Identification of novel genes coding for small expressed RNAs. Science. 2001;294:853–8.
Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005;120:15–20.
Li Y, Zhang M, Chen H, et al. Ratio of miR-196s to HOXC8 mRNA correlates with breast cancer cell migration and metastasis. Cancer Res. 2010;70:7894–904.
Li Z, Huang H, Chen P, et al. miR-196b directly targets both HOXA9/MEIS1 oncogenes and FAS tumour suppressor in MLL-rearranged leukaemia. Nat Commun. 2012;3:688.
Li X, Yang W, Ye W, et al. microRNAs: novel players in hepatitis C virus infection. Clin Res Hepatol Gastroenterol. 2014;38:664–75.
Liu J, Valencia-Sanches AA, Hannon GJ, et al. MicroRNA-depended localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol. 2005;7:719–23.
Liu B, Xiang Y, Zhang H-S. Circulating microRNA-196aa as candidate diagnostic biomarker for chronic hepatitis C. Mol Med Rep. 2015;12:105–10.
Ludwig N, Nourkami-Tutdibi N, Backes C, et al. Circulating serum miRNAs as potential biomarkers for nephroblastoma. Pediatr Blood Cancer. 2015. doi:10.1002/pbc.25481 [Epub ahead of print].
Maru DM, Singh RR, Hannah C, et al. MicroRNA-196a is a potential marker of progression during Barrett's metaplasia-dysplasia-invasive adenocarcinoma sequence in esophagus. Am J Pathol. 2009;174:1940–8.
Masaki S, Ohtsuka R, Abe Y, et al. Expression patterns of microRNAs 155 and 451 during normal human erythropoiesis. Biochem Biophys Res Commun. 2007;364:509–14.
Mekky RY, El-Ekiaby NM, Hamza MT, et al. Mir-194 is a hepatocyte gate keeper hindering HCV entry through targeting CD81 receptor. J Infect. 2015;70:78–87.
Meredith LW, Wilson GK, Fletcher NF, et al. Hepatitis C virus entry: beyond receptors. Rev Med Virol. 2012;22:182–93.

Messina JP, Humphreys I, Flaxman A, et al. Global distribution and prevalence of hepatitis C virus genotypes. Hepatology. 2015;61:77–87.
Monteys AM, Spengler RM, Wan J, et al. Structure and activity of putative intronic miRNA promoters. RNA. 2010;16:495–505.
O'Connell RM, Taganov KD, Boldin MP, et al. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci USA. 2007;104:1604–9.
Okamoto H, Kurai K, Okada K, et al. Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology. 1992;188:331–41.
Osaka E, Kelly AD, Spentzos D, et al. MicroRNA-155 expression is independently predictive of outcome in chordoma. Oncotarget. 2015;6:9125–39.
Pedersen IM, Cheng G, Wieland S, et al. Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature. 2007;449:919–22.
Pestova TV, Shatsky IN, Fletcher SP, et al. A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. Genes Dev. 1998;12:67–83.
Polioudakis D, Bhinge AA, Killion PJ, et al. A Myc-microRNA network promotes exit from quiescence by suppressing the interferon response and cell-cycle arrest genes. Nucleic Acids Res. 2013;41:2239–54.
Popovic R, Riesebeck LE, Velu CS, et al. Regulation of mir-196b by MLL and its overexpression by MLL fusion contributes to immortalization. Blood. 2009;113:3314–22.
Rai D, Karanti S, Jung I, et al. Coordinated expression of microRNA-155 and predicted target genes in diffuse large B-cell lymphoma. Cancer Genet Cytogenet. 2008;181:8–15.
Rebucci M, Sermeus A, Leonard E, et al. miRNA-196b inhibits cell proliferation and induces apoptosis in HepG2 cells by targeting IGF2BP1. Mol Cancer. 2015;14:79.
Riad SE, EL-Akiaby N, Mekky RH, et al. Expression signature of microRNA-155 in hepatitis C virus genotype 4 infection. Biomed Rep. 2015;3:93–7.
Rodriguez A, Vigorito E, Clare S, et al. Requirement of bic/microRNA-155 for normal immune function. Science. 2007;316:608–11.
Seddiki N, Brezar V, Ruffin N, et al. Role of miR-155 in the regulation of lymphocyte immune function and disease. Immunology. 2013;142:32–8.
Sedger LM. microRNA control of interferons and interferon induced anti-viral activity. Mol Immunol. 2013;56:781–93.
Sendi H, Mehrab-Mohseni M, Foureau DM, et al. MiR-122 decreases HCV entry into hepatocytes through binding to the 3′ UTR of OCLN mRNA. Liver Int. 2015;35:1315–23.
Shin VY, Siu JM, Cheuk I, et al. Circulating cell-free miRNAs as biomarker for triple-negative breast cancer. Br J Cancer. 2015;112:1751–9.
Shirasaki T, Honda M, Shimakami T, et al. MicroRNA-27a regulates lipid metabolism and inhibits hepatitis C virus replication in human hepatoma cells. J Virol. 2013;87:5270–86.
Sidorkiewicz M, Grek M, Jozwiak B, et al. Expression of microRNA-155 precursor in peripheral blood mononuclear cells from hepatitis C patients after antiviral treatment. Acta Virol. 2010;54:75–8.
Simmonds P. Genetic diversity and evolution of hepatitis C virus – 15 years on. J Gen Virol. 2004;85:3173–88.
Simmonds P, Bukh J, Combet C, et al. Consensus proposals for unified system of nomenclature of hepatitis C virus genotypes. Hepatology. 2005;42:962–73.
Sun X, Liu Y, Li M, et al. Involvement of miR-485-5p in hepatocellular carcinoma progression targeting EMMPRIN. Biomed Pharmacother. 2015;72:58–65.
Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006;103:2257–61.
Wang J, Zhang X, Wang L, et al. Downregulation of urinary cell-free microRNA-214 as a diagnostic and prognostic biomarker in bladder cancer. J Surg Oncol. 2015a. doi:10.1002/jso.23937 [Epub ahead of print].

Wang Y, Zhao X, Ye X, et al. Plasma microRNA-586 is a new biomarker for acute graft-versus-host disease. Ann Hematol. 2015b. doi:10.1007/s00277-015-2414-z [Epub ahead of print].

World health Organization. Information from the website: http://www.who.int/mediacentre/factsheets/fs164/en/, updated July 2016.

Yong Q, Cheng JR, Zhao J, et al. MicroRNA-155 regulates interferon-γ production in natural killer cells via Tim-3 signaling in chronic hepatitis C virus infection. Immunology. 2015. doi:10.1111/imm.12463 [Epub ahead of print].

Zaltron A, Spinetti A, Biasi L, et al. Chronic HCV infection: epidemiological and clinical relevance. BMC Inf Dis. 2012;12:S2.

Zeng F-R, Tang L-J, He Y, et al. An update of the role of miRNA-155 in pathogenic microbial infections. Microbes Infect. 2015. doi:10.1016/j.micinf.2015.05.007 [Epub ahead of print].

Zhang Y, Wei W, Cheng N, et al. Hepatitis C virus induced up-regulation of microRNA-155 promotes hepatocarcinogenesis by activating Wnt signaling. Hepatology. 2012;56:1631–40.

Zhang GJ, Xiao H-X, Tian H-P, et al. Upregulation of microRNA-155 promotes the migration and invasion of colorectal cancer cells through the regulation of claudin-1 expression. Int J Mol Med. 2013;31:1375–80.

Zhou G, Lu M, Chen J, et al. Identification of miR-199a-5p in serum as noninvasive biomarkers for detecting and monitoring osteosarcoma. Tumour Biol. 2015. doi:10.1007/s13277-015-3421-3 [Epub ahead of print].

Zhu W, Wilson AT, Mathahs MM, et al. Heme oxygenase-1 suppresses hepatitis C virus replication and increases resistance of hepatocytes to oxidant injury. Hepatology. 2008;48:1430–9.

Serum Alpha-Fetoprotein as a Biomarker in Liver Transplantation

40

Samy Kashkoush, Sherif Saleh, and Walid Elmoghazy

Contents

S. Kashkoush (✉)
Department of Hepatobiliary Surgery and Liver Transplantation, National Liver Institute, Minufiya University, Minufiya, Egypt

Organ Transplant Center, King Abdullah Specialist Children Hospital (KASCH) National Guard, Riyadh, Saudi Arabia
e-mail: samykashkoush@gmail.com; kashkoushs@ngha.med.sa

S. Saleh (✉)
Department of Hepatobiliary Surgery and Liver Transplantation, National Liver Institute, Minufiya University, Minufiya, Egypt
e-mail: drsherifsaleh@hotmail.com

W. Elmoghazy (✉)
Hepatobiliary and Liver Transplant Surgery, Hamad Medical Corporation (HMC), Doha, Qatar
e-mail: moghazyw@gmail.com

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_34

Abstract

Alpha-fetoprotein (AFP) is one of the most widely tested biomarkers in medicine. It has long been used in surveillance and diagnosis of hepatocellular carcinoma (HCC), the second cause of cancer-related death worldwide. Modern imaging modalities have replaced AFP in screening and diagnosis of HCC in the last decade. However, the establishment of liver transplantation as the gold standard treatment for HCC patients brought AFP back to the focus of interest. AFP was thoroughly investigated as a selection criterion for transplant candidates and a potential predictor of posttransplant HCC recurrence and survival. The general conclusion that can be made from all the studies is that high or rising AFP values indicate aggressive tumor biology and correlate with poor differentiation, microvascular invasion, posttransplant HCC recurrence, and reduced survival. Different AFP cutoff values were proposed, with or without being incorporated into transplant selection criteria or prognostic models. Because of the wide range of the proposed AFP cutoff values (from 15 to 1000 ng/nL) and the major diversity of the suggested selection criteria and prognostic models, there is no universal agreement on a specific role for AFP in liver transplantation till now. Prospective validation of AFP roles in large well-conducted randomized trials needs to be performed to come up with definite conclusions that can be applied to clinical practice. Also, multiple new biological and genetic markers are being studied in surveillance and diagnosis of HCC with promising results.

Keywords

Alpha-fetoprotein • Hepatocellular carcinoma • Liver transplantation • Selection criteria • Prediction • Prognosis • Recurrence • Survival • Dropout • Down-staging

List of Abbreviations

AASLD	American Association for the Study of Liver Diseases
AFP	Alpha-fetoprotein
AFP-L3	Lens culinaris agglutinin-reactive alpha-fetoprotein
APASL	Asian Pacific Association for the Study of the Liver
CT	Computerized tomography
DCP	Des-gamma carboxyprothrombin
DeMELD	Dropout equivalent MELD
EASL	European Association for the Study of the Liver
HCC	Hepatocellular carcinoma
kDa:	Kilodalton
LRT	Locoregional treatment
MELD	Model for end-stage liver disease
mRECIST	Modified response evaluation criteria in solid tumors
MRI	Magnetic resonance imaging
NAS	Natural AFP slope
NCCN	National comprehensive cancer network
PEI	Percutaneous ethanol injection
PIVKA-II	Protein induced by vitamin K absence or deficiency
RFA	Radio-frequency ablation
TACE	Transarterial chemoembolization
TARE	Transarterial radio-embolization
TTD	Total tumor diameter
TTV	Total tumor volume
UCSF	University of California San Francisco
UNOS	United Network for Organ Sharing
US	Ultrasound

Key Facts About Serum Alpha-Fetoprotein (AFP)

- AFP is a glycoprotein that consists of a single polypeptide chain.
- AFP is the main plasma protein of the human fetus.
- AFP is synthesized in the liver and yolk sac of the human fetus.
- Expression of AFP gene in adults is abnormal and means a pathological condition
- An elevated AFP level in a patient with a liver mass does not necessarily mean the presence of an HCC.
- Up to 40% of patients with small HCC have normal AFP levels.
- AFP is no longer recommended as a diagnostic test for HCC in many of the recent guidelines.

- A high AFP level is a surrogate marker for microvascular invasion, poor differentiation, and aggressive HCC biology.

Definitions

Child-Pugh score	was originally developed to predict surgical outcomes in patients presenting with bleeding esophageal varices; it consists of five clinical features, each of them scored from 1 to 3 (albumin, bilirubin, prothrombin time prolongation, ascites, and encephalopathy) and is used to assess the degree of chronic liver disease and cirrhosis.
Hepatocellular carcinoma	is a primary malignant neoplasm derived from hepatocytes, accounting for about 80% of all liver cancers.
Liver cirrhosis	is a slowly progressing disease in which healthy liver tissue is replaced with fibrous tissue, leading to liver failure and multiple complications.
Loco-regional treatments for HCC	are local ablative therapies for treating patients with hepatocellular carcinoma (as percutaneous ethanol injection, thermal ablation, or intra-arterial chemo- or radio-embolization).
Model for end-stage liver disease (MELD)	is a score calculated using creatinine, bilirubin, and INR to measure the severity of liver disease. Initially, it is developed to predict 90 days mortality risk. Currently, it is used to prioritize recipients for cadaveric organ allocation.
Organ allocation	is the process of determining how organs are distributed; it should promote distribution of organs in an equitable, ethical, and medical sound manner.
Radio-frequency ablation (RFA)	is using the resistive heat resulting from medium frequency alternating electrical current delivered to the tumor through special needles to produce local hyperthermia and tissue destruction.
Screening	occurs when the patient is asymptomatic but undergoes testing in order to detect the disease early before development of symptoms.
Sensitivity of a clinical test	is the ability of the test to correctly identify those patients with the disease.
Specificity of a clinical test	is the ability of the test to correctly identify those patients with no disease.

Surveillance	is the process of serial application of the screening test to detect the disease before it becomes clinically evident.
Transarterial chemoembolization (TACE)	consists of placement of an intra-arterial catheter in the vessels supplying the tumor, to deliver high concentrations of a chemotherapeutic agent (e.g., doxorubicin) along with an embolic agent, such as lipiodol to achieve both targeted chemotherapy and deprivation of tumor arterial supply.

Introduction

Alpha-fetoprotein (AFP) is one of the most widely tested biomarkers in medicine. It has long been used for surveillance and diagnosis of hepatocellular carcinoma (HCC), in addition to several other pathological conditions. Recent advances in imaging technology, which enabled earlier detection and more accurate diagnosis of smaller HCC lesions, and establishment of liver transplantation as the gold standard treatment for selected HCC patients have both led to a switch of AFP utilization from a surveillance and diagnostic test for HCC to a selection and prognostic tool for liver transplant candidates with HCC. Researchers have been studying other HCC-related biomarkers including lens culinaris agglutinin-reactive fraction of AFP (AFP-L3) and des-γ-carboxy prothrombin (DCP), in addition to new genetic markers. However, the roles of these markers in clinical practice still need to be defined.

This chapter will review the changing role of AFP in management of HCC patients with a particular focus on the recent evidence related to its significance in the era of liver transplantation.

AFP Synthesis and Structure

Alpha-fetoprotein (AFP) is the main plasma protein found in the human fetus. It was first detected in the sera of human fetuses in 1956 by Bergstrand and Czar (Adinolfi et al. 1975). The liver and yolk sac are the two major sites for AFP synthesis during fetal development (Gitlin et al. 1972). AFP is believed to be the fetal type of serum Albumin as they are closely related both genetically and structurally, having extensive homologies in amino acid sequence (Deutsch 1991). The genes coding for both proteins have been localized to chromosome 4q (Harper and Dugaiczyk 1983). The AFP concentration in fetal serum is greatest at about 13 weeks of gestation and then starts to decrease while albumin synthesis increases. After birth, serum AFP concentration declines rapidly until it finally reaches the trace concentration found in

normal adults (<10 μg/L) usually by the end of the first year (Nikolic 1992). AFP is a glycoprotein with a molecular mass of about 64 kDa. It consists of a single polypeptide chain and contains 3.4% carbohydrate (Adinolfi et al. 1975).

Pathological Conditions with Elevated AFP

Normally, AFP gene is not expressed in adults except in certain pathological conditions, the most common of which are hepatocellular carcinoma (HCC) and germ-cell tumors as teratoblastoma or embryonal carcinoma of the testis or ovary. Patients with hepatic metastasis from gastrointestinal tumors as gastric, pancreatic, or colonic cancers may also have high AFP levels. A number of genetically determined disorders as hereditary tyrosinemia, ataxia-telangiectasia, and cystic fibrosis are associated with high AFP levels as well (Adinolfi et al. 1975).

Hepatocellular Carcinoma

Liver cancer (including HCC and intrahepatic cholangiocarcinoma) is the second cause of cancer-related death worldwide, and its incidence is still increasing (Bruix et al. 2015). The numbers of estimated new cases and estimated deaths because of liver cancer worldwide were almost similar in 2008, reflecting the high case fatality and aggressive nature of this disease (Jemal et al. 2011). HCC represents the main histological type of primary liver cancer – accounting for 70–85% of the total liver cancer burden – and 80% of those cases are due to chronic hepatitis B and C viral infection (Perz et al. 2006). Hepatitis C virus (HCV)-related HCC was recently reported to be the fastest rising cause of cancer-related death in the United States (El-Serag and Kanwal 2014). Other risk factors in western countries include alcohol-related cirrhosis and possibly nonalcoholic fatty liver disease (NAFLD) associated with obesity. Heavy exposure to aflatoxin B1, in parts of Africa and Asia, is another known risk factor (Jemal et al. 2011).

Surveillance and Diagnosis of HCC

Early detection of HCC allows potentially curative treatment options leading to better patient outcome and prolonged survival (Bruix et al. 2016; Fig. 1). So, surveillance protocols were developed for high-risk patient groups, including those with chronic viral hepatitis or liver cirrhosis related to viral infection. Previously, the majority of HCC patients had advanced disease at presentation, and their AFP levels were mostly elevated. AFP measurement, therefore, was not only used as a diagnostic test for HCC, but it was also implemented as a screening test in surveillance protocols for early detection of HCC (Gebo et al. 2002). In the absence of specific recommendations, regular follow-up of those patients by hepatic ultrasound and serum AFP used to be the most widely adopted surveillance protocol for HCC (Gebo et al. 2002). Recent

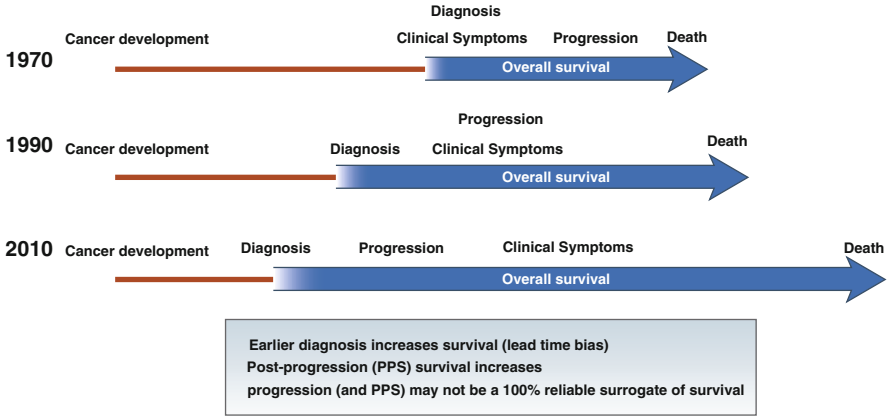

Fig. 1 Changes in diagnosis and treatment of HCC over time. Years ago, imaging techniques were not available. So, HCC was mostly diagnosed when symptomatic progression was close to death. Years later, HCC could be diagnosed at earlier stages, before symptoms developed. However, survival didn't improve much because of absence of effective treatment options. Nowadays, patients are diagnosed with early-stage disease due to surveillance programs, and the best therapy can be selected for each patient, increasing survival times (Modified with permission from Bruix et al. (2016))

studies, however, have shown that both the sensitivity and specificity of AFP are inadequate for effective surveillance and diagnosis of HCC (Lok et al. 2010).

AFP Sensitivity and HCC Surveillance

Up to 40% of patients with small HCC have normal AFP levels. The sensitivity of AFP in those patients would be only 60%, that is, obviously not sensitive enough for a screening test (Lok et al. 2010; Tateishi et al. 2008; Song et al. 2014). Also, measurement of AFP was found to provide no additional benefit to ultrasound surveillance, which showed significantly higher sensitivity for early HCC when performed every 6 months instead of annually (Singal et al. 2009). As such, recent guidelines of the National Comprehensive Cancer Network (NCCN) (Benson et al. 2014), the European Association for the Study of the Liver (EASL) (European Association for the Study of the Liver and European Organisation for Research and Treatment of Cancer 2012), and the American Association for the Study of Liver Diseases (AASLD) (Bruix et al. 2011) have all excluded AFP from HCC surveillance protocols and recommended HCC screening by hepatic US only every 6 months (Song et al. 2016). On the contrary, the Asian Pacific Association for the Study of the Liver (APASL) guidelines, the Chinese, and the Japanese guidelines still recommend the combination of AFP and US for HCC screening. Thus, exclusion of AFP measurement from surveillance of HCC is not universally agreed upon and may need to be further investigated (Song et al. 2016).

AFP Specificity and HCC Diagnosis

High AFP levels have long been considered diagnostic of HCC with high specificity. Recent data, however, suggest that its specificity – and consequently its value as a diagnostic test – is lower than what was previously believed (Tateishi et al. 2008; Bruix et al. 2011). Mild to moderate elevation of serum AFP occurs frequently in chronic liver disease, particularly chronic hepatitis C patients (Sterling et al. 2012). AFP can also be elevated in metastatic colorectal cancer (Sato et al. 1994), gastric carcinoma (Adachi et al. 2003), and intrahepatic cholangiocarcinoma, which is also more common in cirrhotics than in noncirrhotics (Bruix et al. 2011). Therefore, an elevated AFP level in a patient with a liver mass may be suggestive of – but does not necessarily mean – the presence of an HCC (Bruix et al. 2011). So, AFP is no longer recommended as a diagnostic test for HCC in many of the recent guidelines including the AASLD, EASL, and NCCN which rely mainly on the typical radiological features for diagnosis of HCC. AFP, however, remains an adjunctive tool for HCC diagnosis in Asian guidelines, as the APASL and Chinese guidelines (Song et al. 2016).

The typical contrast enhancement pattern in a four-phase dynamic cross-sectional study (enhancement in the arterial phase followed by washout in the portovenous or delayed phases) confirms the diagnosis of HCC (Bruix et al. 2011; Fig. 2). The latest AASLD guidelines recommend that a lesion smaller than 1 cm should be followed up closely at 3 months intervals using the same imaging modality that first showed the lesion. For lesions larger than 1 cm in diameter, further investigations should be performed with a four-phase dynamic study, either CT scan or MRI. If the appearance is not typical for HCC and does not suggest hemangioma, the other dynamic study should be performed or biopsy of the lesion is indicated (Bruix et al. 2011; Fig. 3).

Staging and Management of HCC

Most of the HCC patients have chronic liver disease, and a good percent of them will die from their failing liver rather than the HCC itself. This complicates the picture when considering treatment options. Proper staging is therefore crucial for successful management of HCC patients. The Barcelona Clinic Liver Cancer (BCLC) staging system has been repeatedly validated and widely adopted by most transplant centers (Fig. 4). It links prognosis and treatment options to the main variables that affect the patient's condition including cancer stage, severity of liver disease, performance status, and other comorbidities.

Liver Transplantation for HCC

Surgical resection and liver transplantation are the potentially curative treatment options for HCC (Agopian et al. 2015a). When indicated, liver transplantation is considered the gold standard treatment for patients having HCC and chronic liver disease as it is the only treatment that can address both the tumor and the liver disease

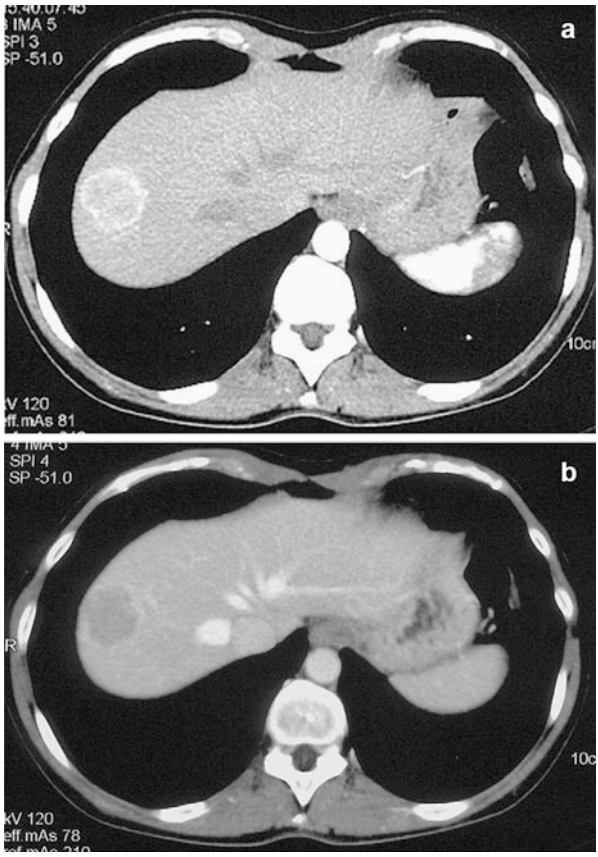

Fig. 2 Dynamic CT scan of a patient with HCC. Typical diagnostic pattern of HCC with contrast enhancement in the arterial phase (**a**) and washout in the portovenous phase (**b**) (Modified with permission from Golfieri et al. (2007))

at the same time (Hakeem et al. 2012). Any other treatment for HCC, even if complete tumor control is achievable, leaves the patient with the problem of a decompensating oncogenic diseased liver that will eventually fail and/or form new HCC lesions (Berry and Ioannou 2013). Liver transplantation for properly selected patients yields an excellent outcome, with more than 70% 5-year survival rates (Bruix et al. 2011).

Evolution of Transplant Indication and Candidate Selection for HCC Patients

The indication of liver transplantation in HCC patients has extremely evolved in less than two decades from being a contraindication in many centers in mid-1990s, until it became a priority deserving exceptional points under the model for end-stage liver disease (MELD) system for organ allocation in 2002 (Yao et al. 2007).

The outcome of early experience with liver transplantation for patients with HCC was generally poor with a 3-year survival rate of 31% (Bismuth et al. 1993). Bismuth et al. 1993 reported significantly better outcome with liver transplantation compared to

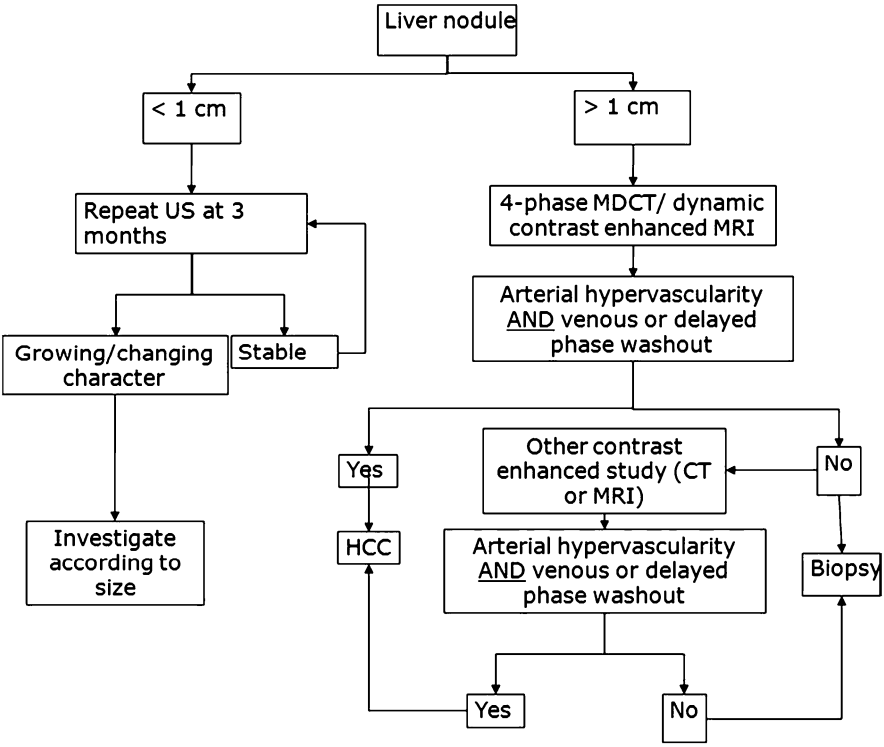

Fig. 3 Diagnostic algorithm for suspected HCC. *CT* computed tomography, *MDCT* multidetector CT, *MRI* magnetic resonance imaging, *US* ultrasound (Adopted with permission from Bruix et al. (2011))

surgical resection for HCC patients with limited tumor load, defined as single or double nodules less than 3 cm in diameter (Bismuth et al. 1993). The landmark study of Mazzaferro et al. (1996) reported excellent outcomes with liver transplantation for selected cirrhotic patients having small unresectable HCC lesions. Posttransplant survival of those patients equaled survival of non-HCC liver transplant recipients. Their selection criteria, known later as Milan criteria, included a single HCC nodule $\leq$5 cm in diameter or up to three tumor nodules; each is $\leq$3 cm with no evidence of macrovascular invasion or extrahepatic disease (Mazzaferro et al. 1996). Milan criteria not only got widely accepted and implemented by many transplant centers worldwide but also became the gold standard selection criteria for liver transplant candidates with HCC, to which any proposed modification needs to be compared (Mazzaferro et al. 2011).

Expansion of Transplant Selection Criteria for HCC

In spite of the general agreement on Milan criteria by many transplant centers across the world since its proposal till now, a number of studies suggested that Milan criteria could be very restrictive denying transplant access to many HCC patients

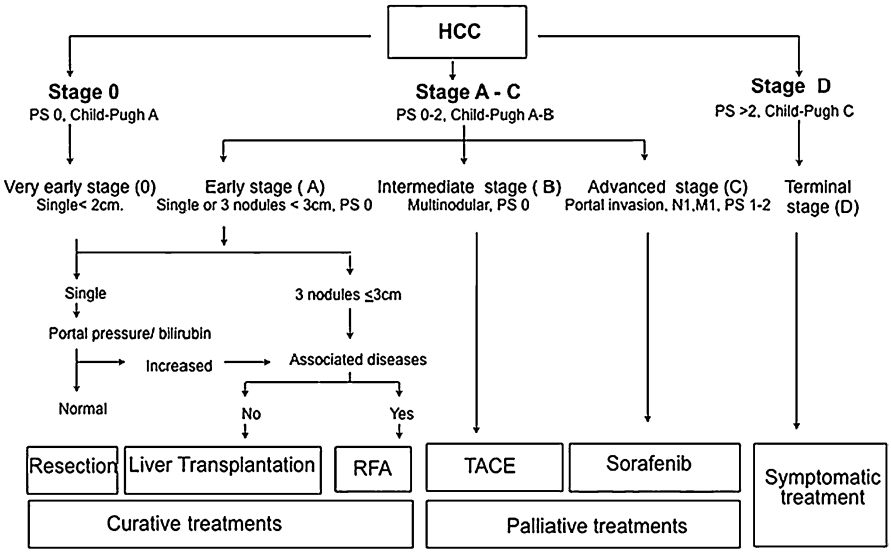

Fig. 4 The BCLC staging system for HCC. *M* metastasis classification, *N* node classification, *PS* performance status, *RFA* radiofrequency ablation, *TACE* transarterial chemoembolization (Adopted with permission from Bruix et al. (2011))

who may otherwise have a reasonably good chance of posttransplant survival (Llovet et al. 1999; Mazzaferro et al. 2009; Yao et al. 2001; Duvoux et al. 2012). Also, the estimated 10–15% under staging resulting from the less accurate imaging modalities at the time Milan criteria were proposed would allow some expansion of the morphologic criteria, having the currently available more precise radiologic scans (Bruix et al. 2011). Accordingly, several more permissive transplant selection criteria were proposed by different centers for HCC patients. Those proposed criteria suggested variable extensions of the morphological characteristics of the tumor, whether nodule number, maximum diameter, or both (Kashkoush et al. 2014). Among the extended selection criteria that were validated and accepted were the University of California, San Francisco (UCSF) (Yao et al. 2001) and the up-to-seven criteria (Mazzaferro et al. 2009). Multiple other criteria were proposed, but discussing them is not within the scope of this review.

UCSF Criteria

The UCSF criteria include patients with solitary tumors $\leq$6.5 cm, or those having three or fewer tumors, the largest $\leq$4.5 cm with total tumor diameter (TTD) $\leq$8 cm, and no macrovascular invasion (Yao et al. 2001). Excellent posttransplant outcome of HCC patients meeting those UCSF criteria was reported with 5-year survival of 75%. However, being retrospectively derived from explant pathology, UCSF criteria were considered cautiously until prospectively validated and showed similar

predictive ability for recurrence and survival compared to Milan criteria in spite of including up to 20% more candidates for transplantation (Yao et al. 2007).

Up-To-Seven Criteria

Mazzaferro et al. (2009) proposed other extended selection criteria known as the up-to-seven criteria where seven is the sum of the largest tumor size in cm and the number of tumors (Mazzaferro et al. 2009). The study included 1556 patients transplanted for HCC at 36 transplant centers (31 in Europe, 4 in the United States, and 1 in Asia). Patients outside Milan but meeting the up-to-seven criteria had 5-year posttransplant survival rate similar to patients within Milan if there was no evidence of microvascular invasion on explant pathology (71.2% vs. 73.3%) and a significantly lower 5-year survival (53.6%) if microvascular invasion was detected. Free online software called the Metroticket calculator was developed to calculate 3- and 5-year survival probability for potential candidates (Fig. 5). The calculator can also provide three survival estimates in relation to vascular invasion, whether present, absent, or unspecified. It is therefore clear that the major limitation of the model is the unavailability of information on microvascular invasion before transplantation. So, a surrogate preoperative, noninvasive marker of microvascular invasion is very crucial for implementing this model.

Fair Organ Allocation Mandates Reliable Prediction

Whether to adopt restrictive or permissive selection criteria depends mainly on availability of organs and dynamics on the transplant waiting list (Clavien et al. 2012). The imbalance between the number of potential liver transplant recipients and the available organ donors will continue to worsen (Berry and Ioannou 2013). This relative organ shortage necessitates optimization of the organ allocation process so that maximum benefit of the limited organ pool is achieved. A recent international consensus conference has recommended that: "Liver transplantation should be reserved for HCC patients who have a predicted 5-year survival comparable to non-HCC patients" (Clavien et al. 2012). It is generally accepted that HCC patients with an anticipated 5-year survival less than 50% should not be considered for liver transplantation (Zimmerman et al. 2008). To fairly apply this rule, reliable prognostic data are needed before declining patients' access to transplantation.

Tumor Biology: A Major Determinant of Treatment Outcome

Selection criteria depending on tumor morphology alone are generally crude predictors of outcome. While being the gold standard selection criteria, a number of patients exceeding Milan will have good prognosis if transplanted, and a subgroup of

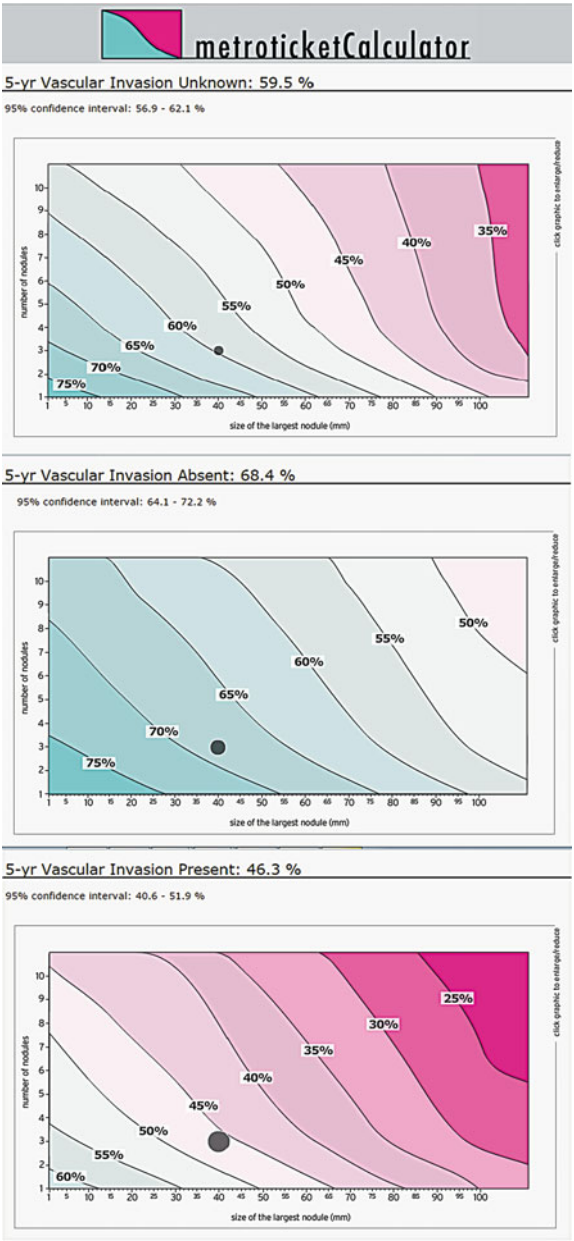

Fig. 5 Metroticket survival calculator for transplant candidates with HCC. An example of survival estimates generated by the free online Metroticket calculator for a patient with three HCC lesions; the largest is 4 cm in diameter. The calculator provides three survival estimates in relation to vascular invasion, whether unspecified (*upper*), absent (*middle*), or present (*lower*). This reflects the importance of AFP being a known surrogate marker for vascular invasion

patients within Milan will develop HCC recurrence and have poor prognosis (Hakeem et al. 2012; Duvoux et al. 2012). This could be explained by more than one fact. First, the accuracy of radiological tests will affect the predictive value of Milan and other related criteria that depend on morphological features of the tumor alone. Secondly, and more importantly, these criteria don't account for tumor biology, which is a major determinant of disease course and outcome (Clavien et al. 2012).

Features of aggressive tumor biology including microvascular invasion, high grade (poor differentiation), and satellite nodules, have been reported by many workers as independent predictors of HCC recurrence and poor survival following liver transplantation (Hakeem et al. 2012; Jonas et al. 2001; Moya et al. 2002; Duffy et al. 2007; Vibert et al. 2010). Those are microscopic features diagnosed only upon histological examination of explanted livers and cannot be definitely confirmed during the pretransplant stage (Gouw et al. 2011). Information related to tumor biology that can be obtained from a needle biopsy is usually incomplete, in addition to the documented risk of tumor seeding along the biopsy tract (Takamori et al. 2000). Therefore, presence of surrogate markers of aggressive tumor biology and poor histological features that can be measured preoperatively would be very valuable predictors of treatment outcome better than imaging-based selection criteria. AFP was thought to play that role, and it has been repeatedly studied in this regard. High-serum AFP expression correlates with extensive cellular proliferation, angiogenesis, and limited apoptosis (Song et al. 2016). AFP was found to be an independent predictor of both microvascular invasion and tumor differentiation; both of which are associated with disease recurrence and poor prognosis (Hakeem et al. 2012; Vibert et al. 2010).

AFP as a Predictor of HCC Recurrence and Survival After Liver Transplantation

AFP is the most widely tested biomarker for HCC (Lai et al. 2012a). A large number of studies have reported AFP significance as a predictor of disease recurrence and survival following liver transplantation for HCC (Hakeem et al. 2012; Clavien et al. 2012). Different AFP parameters were investigated including the highest absolute value, the latest value before transplant, and the dynamic AFP changes while on the waiting list. Other reports highlighted the significance of AFP as a predictor of patient dropout and/or success of pretransplant LRT. Also the response of AFP to locoregional treatment (LRT) and down-staging was investigated as a possible predictor of posttransplant outcome (Lai et al. 2013).

Significance of Absolute AFP Cutoff Values before Liver Transplantation

Several publications have reported different AFP cutoff values and their significant association with posttransplant HCC recurrence and poor survival. A recent systematic review and meta-analysis of the published studies has shown that AFP levels

>1000 ng/mL are associated with poor disease-free and overall survival, microvascular invasion, and poor differentiation. However, due to the heterogeneity of the included studies and the widely variable AFP cutoff values reported, which ranged from 20 to >1000 ng/mL, no valid statistical meta-analysis could be performed and no firm conclusions could be reached (Hakeem et al. 2012).

Hameed et al. 2014 reviewed 211 consecutive liver transplant recipients meeting Milan criteria, trying to establish an AFP cutoff for exclusion of candidates at high risk of HCC recurrence among this homogeneous group of patients (Hameed et al. 2014). The recorded AFP levels were the last ones within 3 months before liver transplantation. Vascular invasion was found to be the only significant variable predicting posttransplant HCC recurrence. However, AFP level >1000 ng/mL was the strongest pretransplant predictor of vascular invasion. The 5-year disease-free survival was 53% for patients with AFP levels >1000 ng/mL compared to 80% for patients with AFP levels ≤1000 ng/mL ($P = 0.026$). Interestingly, the significance of AFP cutoff >1000 ng/mL as a predictor of poor posttransplant outcome was reported by earlier studies from the same center. Proposing UCSF criteria, Yao et al. (2001) reported that an AFP level >1000 ng/mL was a predictor of reduced survival by univariate analysis only (Yao et al. 2001). Validating UCSF criteria, Yao et al. (2007) again reported the significance of AFP >1000 ng/mL as a predictor of HCC recurrence by both univariate and multivariate analyses (Yao et al. 2007). However, clinical application of this finding was not suggested at that time.

The AFP >1000 ng/mL cutoff was also reported by two large multicenter studies from Japan and France as a significant predictor of posttransplant HCC recurrence and poor survival (Duvoux et al. 2012; Todo et al. 2007). Both studies stratified the AFP cutoff into two values, the higher being >1000 ng/mL and the lower was either 200 (Todo et al. 2007) or 100 ng/mL (Duvoux et al. 2012). The 2 studies proposed new criteria/model for candidate selection (described later). The study of Todo et al. (2007) included 653 HCC patients who received living donor liver transplant at 49 transplant centers in Japan and reported significantly worse 1-, 3-, and 5-year overall survival of 64.9%, 42.5%, and 34% for patients with AFP levels ≥1000 ng/mL, compared to 83.8%, 77.3%, and 72.2% for patients with AFP ≤200 ng/mL (Todo et al. 2007). The study of Duvoux et al. involved 972 HCC patients transplanted at 21 centers in France and reported significantly higher 5-year HCC recurrence rate of 53% and worse 5-year overall survival of 39% for patients with AFP levels >1000 ng/mL compared to 16% and 67.5% for patients with AFP ≤100 ng/mL, respectively (Duvoux et al. 2012).

The significance of the AFP >1000 ng/mL cutoff value in relation to posttransplant tumor recurrence and poor survival was also reported by other workers (Lao et al. 2009; Zou et al. 2008). Zou et al. (2008) also showed an association between AFP levels >1000 ng/mL and early fatal recurrence of HCC within 1 year after liver transplantation (Zou et al. 2008).

Other lower absolute AFP cutoff values were suggested by different studies. An AFP cutoff value of 400 ng/mL was suggested by more than one researcher (Toso et al. 2009; Mailey et al. 2011; Merani et al. 2011) (see later). AFP >300 ng/mL was found among other variables to be significantly associated with HCC recurrence

after transplantation (Shetty et al. 2004). An AFP >200 ng/mL was reported by DuBay et al. (2011), to be significantly associated with dropout and lower posttransplant survival (DuBay et al. 2011).

Large AFP Studies Based on Transplant Registry Data

The huge database of the transplant registry of the United States, the United Network for Organ Sharing (UNOS), was analyzed by a number of researchers trying to define a specific role for AFP in liver transplantation for HCC. The conclusions of these studies drive their significance from being based on a very large sample of patients. The main disadvantage, however, is that data on HCC recurrence are missing with overall survival being reported instead (Hameed et al. 2014).

More than one study specifically recommended an AFP cutoff value of 400 ng/mL (Toso et al. 2009; Mailey et al. 2011; Merani et al. 2011) including the study of Toso et al. (2009) on 6478 transplant recipients that also proposed new selection criteria formed of AFP cutoff value of 400 ng/mL and total tumor volume (TTV) (Toso et al. 2009) (see later). Mailey et al. (2011) stratified the studied 2253 HCC transplant recipients into 3 AFP groups: low (<20 ng/mL), intermediate (20–399 ng/mL), and high (≥400 ng/mL). The intermediate and high AFP groups had significantly worse overall survival compared to the low AFP group, which was similar to that of patients with nonmalignant disease. Patients with AFP levels ≥400 ng/mL had significantly lower 1-, 3-, and 5-year overall survival of 82%, 63%, and 52% compared to 92%, 82%, and 74% for those with AFP <20 ng/mL ($P < 0.001$) (Mailey et al. 2011).

Merani et al. (2011) studied 6817 HCC patients and found that the last AFP level before transplantation was an independent predictor of overall survival ($p \leq 0.001$) and that an AFP ≤400 ng/mL was associated with favorable survival, irrespective of the original AFP level (even if originally >1000 ng/mL) (Merani et al. 2011).

A notable correlation between AFP and post-transplant survival was reported by the study of (Berry et al. 2013), which included 45,267 first-time liver transplant recipients and showed that progressive rise of pre-transplant AFP levels correlated with progressive worsening of post-transplant survival. Whereas patients without HCC had 6-year posttransplant survival of 72%, those with HCC had 6-year survival of 70% (if AFP was 0–5 ng/mL), 60% (if AFP was 16–65 ng/mL), 57% (if AFP was 66–320 ng/mL), or finally 51% (if AFP was >320 ng/mL). The study concluded that serum AFP, rather than tumor burden, was the preoperative variable most significantly associated with posttransplant survival. Moreover, patients exceeding Milan criteria had excellent posttransplant survival if their serum AFP levels were low (0–15 ng/mL), whereas those meeting Milan criteria had poor survival if their AFP levels were higher (≥66 ng/mL) (Berry and Ioannou 2013).

Dynamic AFP Changes Before Transplantation (The AFP Slope)

A number of studies investigated the possible significance of AFP dynamics before liver transplantation predicting posttransplant HCC recurrence and survival. Han et al. (2007) specifically studied AFP slope before liver transplantation in 48 patients transplanted for HCC (Han et al. 2007). They found that although the absolute value of AFP was not a predictor of recurrence, AFP slope was the only preoperative independent predictor of HCC recurrence. AFP slope also correlated significantly with large tumor diameter >7 cm and vascular invasion. Patients with a preoperative AFP slope >50 μg/L/month had significantly lower 1-year disease-free survival than those with an AFP slope ≤50 μg/L/month (40% vs. 90%, $P < 0.001$). The same center published a more recent study including 144 patients transplanted for HCC. They found that both rising natural AFP slope (NAS) (>0.1 μg/L/day) and Milan criteria were significant predictors of HCC recurrence. A rising NAS was also a significant predictor of microvascular invasion, a well-known risk factor for HCC recurrence (Dumitra et al. 2013).

The same concept was investigated by Vibert et al. (2010), where AFP progression was defined as an increase of serum AFP more than 15 μg/L/month. Significant 5-year overall and recurrence-free survival differences were found between patients with and without AFP progression (54% and 47% vs. 77% and 74% respectively, $P = 0.01$). AFP progression also significantly correlated with vascular invasion and satellite nodules on histological examination of the explanted livers. The study concluded that preoperative AFP progression >15 μg/L/month (equivalent to 15 ng/mL) was not only the most relevant prognostic factor to HCC recurrence and poor survival but also an independent predictor of unfavorable pathological features, which are themselves significant risk factors for HCC recurrence (Vibert et al. 2010).

Again, the AFP slope >15 ng/mL/month was reported by Lai et al. (2013) in a large multicenter study as a significant risk factor for posttransplant HCC recurrence and reduced survival, irrespective of the Milan status. The study included 422 HCC patients (306 within and 116 outside Milan criteria) who underwent LRT then liver transplant at six European centers between 1999 and 2010. In addition to the AFP slope, radiological progression of tumors according to the modified Response Evaluation Criteria in Solid Tumors (mRECIST) was found to be another significant predictor of HCC recurrence and death for all patients, whether within or outside Milan criteria (Lai et al. 2013). A controversial issue in calculating AFP slope was the two time points at which AFP was measured. Whereas the last pretransplant AFP value was clearly a significant point, it was not certain what is the best other time point to consider, given the fluctuations of AFP values with or without LRT. Therefore, the same group published a recent study investigating the AFP "delta-slope" (obtained by calculating the delta value between the two different AFP slopes, before and after LRT). The study retrospectively reviewed 124 candidates transplanted for HCC between 2004 and 2012 and reported that the AFP delta-

slope was also an independent predictor of posttransplant HCC recurrence and intent-to-treat survival. The 5-year intent-to-treat and recurrence-free survival rates were 66% and 92% versus 37% and 54%, for patients meeting and exceeding the AFP delta-slope cutoff value of 15 ng/mL/month, respectively (Lai et al. 2015).

Clearly, there is sufficient evidence to suggest that a rising AFP level before transplantation is a predictor of aggressive biology, posttransplant HCC recurrence, and poor outcome. However, the suggested AFP slope (and delta-slope) of 15 ng/mL/month still needs to be prospectively validated.

Transplant Selection Criteria Combining AFP and Tumor Morphology

Since serum AFP has emerged as a possible predictor of posttransplant survival in HCC patients, several studies have investigated combining it with different HCC morphologic criteria for better selection of transplant candidates in the current era of organ shortage (Berry and Ioannou 2013). The common objective of those studies is to optimize organ allocation so that candidates with expected better prognosis may get access to the limited organs. Even though most of those proposed combinations were shown to be comparable to Milan criteria, there is no universal agreement on any of them to replace Milan, which continues to be the gold standard selection criteria recommended by most guidelines.

AFP was combined with variable morphologic criteria including total tumor diameter (TTD), total tumor volume (TTV) or tumor number, and maximum size. Other studies have also combined AFP with Milan criteria, with or without other biomarkers.

Hangzhou Criteria

Zheng et al. 2008 retrospectively reviewed 195 HCC patients transplanted in China and proposed combined selection criteria (Hangzhou criteria), which consist of either (A) TTD $\leq$8 cm or (B) TTD >8 cm, with AFP $\leq$400 ng/mL and histopathologic grade I or II. The Hangzhou criteria included 27 more patients with no significant survival difference compared to Milan criteria (5-year survival 78% vs. 72%, respectively, $P > 0.05$) (Zheng et al. 2008). Recently, the same transplant center reviewed data of 6012 HCC patients transplanted in China and stratified Hangzhou criteria into type (A) (TTD $\leq$8 cm, or >8 cm but AFP $\leq$100 ng/mL with well to moderate tumor differentiation) and type (B) (TTD >8 cm but AFP between 100 and 400 ng/mL with well to moderate tumor differentiation) (Xu et al. 2015). Type A showed significantly higher 5-year recurrence-free survival rates compared to type B (69.5% vs. 39% for types A and B, respectively, $P < 0.001$). Since the main difference between types A and B is the AFP level, it is clear that high AFP is a significant risk factor for posttransplant HCC recurrence and poor survival.

Limitations of Hangzhou criteria include being based on Chinese population, where hepatitis B is the most prevalent cause of chronic liver disease and HCC complicating those patients tends to have better differentiation than HCC complicating hepatitis C infection (Busuttil 2008). Also the need for tumor biopsy in those criteria is a concern with the potential risks of seeding and/or bleeding (Lai et al. 2012b).

The AFP-TTD Criteria

A recent multicenter study from Italy proposed combined selection criteria more or less similar to Hangzhou criteria, without the need for tumor biopsy. (Lai et al. 2012b) retrospectively reviewed 158 HCC patients transplanted between 1999 and 2008 at the three centers of the Rome Inter-University Consortium for Organ Transplantation. AFP >400 ng/mL and TTD >8 cm were the strongest preoperative predictors of HCC recurrence. Combined AFP-TTD criteria were thus proposed (TTD ≤8 cm and AFP ≤400 ng/mL), which included 22% more candidates than Milan criteria (143 vs. 117 patients) and 7.5% more candidates than UCSF criteria (133 patients). Five-year disease-free survival rates were similar for patients meeting AFP-TTD, Milan, and UCSF criteria (74.4%, 72.9%, and 71.7%, respectively), with no statistically significant differences (Lai et al. 2012b).

The AFP-TTV Criteria

Instead of the TTD, Toso et al. (2009) introduced the principle of total tumor volume (TTV) and proposed composite selection criteria formed of TTV ≤115 cm^3 and AFP ≤400 ng/mL (Toso et al. 2009). As mentioned before, the study was a retrospective analysis of the large transplant registry database of the United States including 6478 recipients. Patients not meeting those criteria had an overall survival below 50% at 3 years.

The devised scoring system of Yang et al. (2007) is an example of combined transplant selection criteria formed of pretransplant AFP level, tumor size, and number (Yang et al. 2007). The study retrospectively analyzed data of 63 HCC patients who received living donor liver transplants in Seoul, Korea, between 1999 and 2005. The score of each parameter ranged from 1–4 points as follows: tumor size, ≤3, 3.1–5, 5.1–6.5, >6.5 cm; tumor number, 1, 2 or 3, 4 or 5, or ≥6 nodules; and AFP, <20, 20.1–200, 200.1–1000, >1000 ng/mL. Applying this scoring system, patients with a score of 3–6 qualify for transplantation and those with a score 7–12 won't. Milan, UCSF, and the proposed scoring criteria all significantly correlated with HCC recurrence and death with similar 3-year survival (80%, 78%, and 79%, respectively). However, the scoring criteria would include more candidates (70% compared to 65% for UCSF and 59% for Milan) while maintaining excellent posttransplant survival.

Table 1 Simplified user-friendly version of AFP model. The score is calculated by adding the individual points for each variable. A score of >2 identifies patients at higher risk of HCC recurrence (Modified with permission from Duvoux et al. (2012))

Variables	Points
Largest diameter, cm	
≤3	0
3–6	1
>6	4
Number of nodules	
1–3	0
≥4	2
AFP level, ng/mL	
≤100	0
100–1000	2
>1000	3

The AFP Model

The AFP model proposed by Duvoux et al. (2012) is another example of transplant selection criteria incorporating AFP with tumor size and number. The model was developed in a large multicenter study in France including 972 adult transplant recipients for HCC divided into two cohorts: training and validation (537 and 435 patients, respectively). The study showed that AFP is an independent predictor of HCC recurrence and it correlates with vascular invasion and tumor differentiation. The proposed model was reported to improve prediction of HCC recurrence and survival compared to Milan criteria. Using a simplified version of the model (Fig. 4), a score is calculated between 0 and 9. A score higher than 2 predicted markedly increased 5-year HCC recurrence risk (50.6% vs. 8.8%, $P < .001$) and decreased survival (47.5% vs. 67.8%, $P < .002$) irrespective of Milan status. A group of patients exceeding Milan had a score of 2 or lower because AFP was <100 ng/mL and had low 5-year HCC recurrence risk (14.4% vs. 47.6% $P = .006$). On the contrary, another group of patients within Milan had a score higher than 2 because AFP was >1000 ng/mL and had a high HCC recurrence risk (37.1% vs. 13.3%; $P < .001$) (Duvoux et al. 2012).

This AFP model was recently validated in a study from Spain including 109 patients transplanted for HCC (Varona et al. 2015). The study showed that the AFP model was a more accurate prognostic tool than other established criteria (including Milan and the up-to-seven criteria) in prediction of HCC recurrence and survival after liver transplantation (Table 1).

Incorporating AFP into Milan Criteria

As mentioned before, high serum AFP >1000 ng/mL was shown by more than one study to be a surrogate marker for vascular invasion and a significant predictor of posttransplant HCC recurrence. Hameed et al. (2014) found that incorporating AFP >1000 ng/mL as an exclusion criterion for patients within Milan would have excluded only 4.7% of patients from liver transplantation, but this could have

reduced HCC recurrence by 20% as well (Hameed et al. 2014). Also, Chaiteerakij et al. (2015) studied AFP and other biomarkers – including DCP and AFP-L 3% – and the value of combining these markers with Milan criteria for optimizing the liver transplant eligibility decision. They found that all biomarkers significantly correlated with HCC recurrence and that the hazard ratio for HCC recurrence was 3.5 for DCP $\geq$7.5 ng/mL and 2.8 for AFP $\geq$250 ng/mL. Combining Milan criteria with the selected AFP cutoff value of $\geq$250 ng/mL significantly increased the hazard ratio for HCC recurrence from 2.6 for being outside Milan criteria alone to 8.6 for the combined Milan/AFP criteria ($P < 0.001$) (Chaiteerakij et al. 2015).

Thus, incorporating pretransplant AFP into Milan criteria may exclude more candidates from liver transplantation, yet it improves the performance of Milan criteria in prediction of HCC recurrence after transplantation. Again, the appropriate cutoff value needs to be determined.

Combining AFP and DCP for Prediction of Transplant Outcome

Since the combination of AFP and DCP (PIVKA II) was reported to increase the sensitivity and specificity for diagnosis of HCC (Yang et al. 2007), it was also investigated as a better prognosticator of transplant outcome for candidates with HCC, either alone or with tumor morphologic criteria.

The A-P Levels

Combined criteria formed of both AFP $\leq$200 ng/mL and PIVKA II $\leq$100 mAU/mL were proposed by Todo et al. (2007) in a large multicenter study that included 653 HCC patients transplanted at 49 centers in Japan between 1989 and 2005. Both pretransplant AFP and PIVKA II (together, referred to as the A-P levels) were found to be independent predictors of HCC recurrence and poor survival by multivariate analysis. Patients within Milan criteria had no significantly different 5-year disease-free survival if they met or exceeded the A-P levels (96.4% vs. 74.7%). Patients beyond Milan criteria but within the A-P levels had favorable 5-year disease-free survival which was significantly better than that of patients beyond both criteria (79% vs. 40%). The combined A-P levels could include about 50% of candidates exceeding Milan criteria while maintaining satisfactory recurrence-free survival (Todo et al. 2007).

Combined AFP, DCP, and Tokyo Criteria

A similar and more recent study from Japan (Shindoh et al. 2014) proposed a combination of the serum AFP and DCP levels with the morphologic Tokyo criteria ($\leq$5 tumors with each tumor $\leq$5 cm). The study reported that presence of at least two of the following three factors including AFP >250 ng/mL and DCP >450 ng/mL and exceeding Tokyo criteria significantly correlated with a worse 5-year disease-

free and overall survival rates (20% vs. 97% and 20% vs. 84%, respectively, $P < 0.001$).

Dropout of Transplant Candidates and Predictive Role of AFP

HCC patients on the transplant waiting list may experience tumor growth beyond the acceptable criteria for transplantation leading to their "dropout" (disqualification for transplantation) (Cucchetti et al. 2011a). Longer waiting time on the transplant list – because of organ shortage – has resulted in overall increase of the dropout rates. The reported dropout risk of HCC patients ranged from 15% to 30% at 1 year (Galuppo et al. 2013). Llovet et al. (1999) compared outcomes of resection and transplantation for HCC and showed that dropout of transplant candidates as a result of long time on the waiting list was the cause behind the lower outcome of the transplant group on an intent-to-treat basis (Llovet et al. 1999). Therefore, prediction of patients who will likely dropout and their prioritization for liver transplantation is crucial for improving transplant outcome.

Interestingly, the same study (Llovet et al. 1999) and a number of other studies have shown that a higher AFP level is an independent predictor of patients' dropout while on the transplant waiting list (Yamashiki et al. 2004; Freeman et al. 2006; Majno et al. 2011; Washburn et al. 2010; Mehta et al. 2013). Yamashiki et al. (2004) found that a baseline AFP $\geq$100 ng/mL was the only factor that significantly correlated with delisting of transplant candidates due to tumor progression (Yamashiki et al. 2004). Washburn et al. (2010) reported a higher dropout rate of 24.9% in patients with AFP >1000 ng/mL which was significantly different from the 7.4% dropout rate of patients with AFP <500 ng/mL (Washburn et al. 2010).

AFP and Dropout Probability Scores

The scoring system called model for end-stage liver disease (MELD) is being used by transplant centers in the United States and elsewhere for prioritization of patients for liver transplantation. Because of the risk of dropout due to tumor progression, HCC patients are currently given exception MELD points to equate their access with non-HCC patients to donor livers (Washburn et al. 2010). Freeman et al. (2006) analyzed risk factors for dropout of candidates with HCC and proposed a dropout risk model by incorporating AFP level and maximum tumor diameter with the calculated MELD score. The proposed HCC-MELD score could predict dropout risk of HCC patients within 90 days of listing, and so patients with higher dropout risk may be prioritized for transplantation (Freeman et al. 2006).

Washburn et al. (2010) compared dropout rates and risk factors for HCC and non-HCC patients and found that – with the exception MELD points – the dropout rate of non-HCC patients was significantly higher than HCC patients ($P < 0.0001$). The study recommended that a scoring model incorporating MELD, AFP, and tumor

size would help prioritize HCC patients with higher risk of dropout and – at the same time – ensure a more equitable liver graft allocation (Washburn et al. 2010).

A national conference on liver allocation for HCC patients in the United States recommended that an HCC priority score needs to be developed, which incorporates the calculated MELD score, serum AFP, tumor size, and rate of growth (Pomfret et al. 2010). Toso et al. (2012) proposed such a model, including tumor number in addition to AFP and tumor size (Toso et al. 2012). This new dropout equivalent MELD (DeMELD) score was validated in two patient cohorts in the United States and United Kingdom and found to provide a dynamic and more equitable estimation of dropout risk than the use of exception MELD points (Toso et al. 2014).

Disadvantage of the HCC Dropout Probability Scores

Adopting HCC dropout probability scores will prioritize patients with higher dropout risk for liver transplant. This may have negative impact on posttransplant outcome since those patients have more advanced HCC, with bigger tumor sizes and higher AFP levels (Cucchetti et al. 2011b). In other words, the higher the dropout probability scores, the higher the predicted tumor recurrence and the lower the posttransplant survival. This is simply because factors affecting dropout risk and posttransplant survival are basically the same (Cucchetti et al. 2011b).

Pretransplant Locoregional Treatment (LRT) for HCC and Their Indications

LRT of HCC lesions in potential transplant candidates, either before listing or while being on the transplant waiting list, has become the standard of care in most transplant centers worldwide (Cescon et al. 2013). There are two main indications for LRT in those patients. The first is to stabilize tumors initially meeting transplant selection criteria in order to prevent or reduce their progression beyond the transplantable limits and consequently their dropout of the transplant waiting list. This strategy is called "bridging," and it is most beneficial for patients with T2 tumors with more than 6 months estimated waiting time on the transplant list (Majno et al. 2011). The second indication is to reduce tumor burden exceeding transplant selection criteria in order to meet those criteria and qualify for liver transplantation. This strategy is called "down-staging," and it was initially recommended by the group from L'Hopital Paul Brousse, Paris, France, in 1997 (Galuppo et al. 2013). A period of follow-up is required to verify response to treatment before considering down-staging successful and listing those patients for transplantation (Cescon et al. 2013).

LRTs include transarterial chemoembolization (TACE), transarterial radioembolization (TARE), radio-frequency ablation (RFA), and percutaneous ethanol injection (PEI) in addition to other new modalities (Toso et al. 2010). TACE and percutaneous ablations are the treatments most frequently used in transplant candidates (Cescon et al. 2013; Toso et al. 2010). In addition to reduction of dropout of

listed candidates and down-staging of noncandidates to qualify for transplant, TACE before liver transplant may have other indications including detection of additional nodules missed by CT scan and ultrasound, prediction of posttransplant disease recurrence, and selection of suitable candidates for transplantation (Cucchetti et al. 2011a; Otto et al. 2006, 2013). The effect of LRT depends on how much pathologic tumor necrosis will be achieved (Agopian et al. 2015b). The reported rates of complete pathologic response ranged from 27% to 57% after TACE and from 47% to 75% after thermal ablation (Agopian et al. 2015b; Pompili et al. 2013).

AFP Response to LRT Predicts Treatment Outcome

AFP has been studied as a possible predictor of response to LRT in HCC patients. Riaz et al. (2009) showed that AFP response (defined as more than 50% decrease from a baseline AFP >200 ng/mL) is a reliable predictor of radiologic response, tumor progression, and overall survival for HCC patients treated with LRT. He also suggested that AFP response was able to predict treatment response earlier than imaging (Riaz et al. 2009). The study however didn't include data on liver transplantation.

Agopian et al. (2015b) studied AFP among other predictive factors of a complete pathologic response in 501 patients with HCC treated with LRT before liver transplantation. He reported that median AFP levels (both maximum and immediate pretransplant values) were significantly lower in patients with complete pathologic response and that reduction of AFP after LRT compared to maximum values was an independent predictor of complete pathologic response, which correlated significantly with recurrence-free survival after liver transplantation (Agopian et al. 2015b).

Bridging Effect of LRT and AFP Predictive Value

Whereas older studies showed no specific impact of LRT on waiting list dropout, the most recent series have shown lower dropout rates between 3.0% and 9.3% with bridging strategies (Pompili et al. 2013). Frangakis et al. (2011) reported a significant reduction of waiting list dropout for HCC patients treated with TACE to 3% versus 15% for untreated patients, respectively ($P = 0.04$). The 2-year survival was better for the TACE group, but it did not reach statistical significance (76.0% vs. 57.3% $P = 0.078$) (Frangakis et al. 2011). The current recommendation is that using LRT for bridging HCC patients on the transplant wait list is not harmful and may be beneficial for patients with T2 lesions and an expected waiting time on the transplant list of more than 6 months (Majno et al. 2011; Pomfret et al. 2010).

AFP measured before or after LRT may predict the success of bridging treatment and dropout risk. (Cucchetti et al. 2011a) reported that nodule number and AFP >400 ng/mL at diagnosis of HCC were the tumor-related factors that significantly correlated with response to bridging therapy at 3 months posttreatment (Cucchetti

et al. 2011a). On the other hand, Mehta et al. (2013) found that an AFP >20 ng/mL after the first LRT is a significant predictor of dropout in multivariate analysis and that the level of AFP significantly correlates with the 1-year cumulative incidence of dropout. The study reported significantly higher dropout rate of 59.5% in patients with AFP >500 ng/mL compared to 12.7% in those with AFP ≤20 ng/mL (Mehta et al. 2013).

AFP and Definition of Down-Staging

Down-staging HCC lesions exceeding Milan or UCSF criteria is a valid alternative to the controversial expansion of the standard selection criteria (Pompili et al. 2013). Different LRTs have been used for down-staging of advanced HCC lesions with variable success rates (Gordon-Weeks et al. 2011). A recent systemic review reported successful down-staging in 24–69% of HCC patients with posttransplant recurrence-free and overall survival rates comparable to patients initially within Milan criteria (Gordon-Weeks et al. 2011). However, authors reported not only variable inclusion criteria for down-staging but also different definitions of successful down-staging. A US-National Consensus Conference recommended eligibility criteria for down-staging to include a single tumor ≤8 cm or 2–3 tumors, each ≤5 cm, with a TTD ≤8 cm and no vascular invasion by imaging studies. The conference also defined successful down-staging as a residual tumor meeting Milan criteria in addition to reduction of serum AFP to <500 ng/mL for those patients with an initial AFP >1000 ng/mL (Pomfret et al. 2010).

AFP Predicts Down-Staging Success

High AFP levels before LRT were reported in a number of studies as a significant predictor of down-staging failure. Barakat et al. (2010) reported significantly higher mean AFP levels in the non-down-staged group of HCC patients compared to the down-staged group (5670 ng/mL vs. 799 ng/mL, $P < 0.048$) (Barakat et al. 2010). Yao et al. (2008) reported successful down-staging in 70.5% of a cohort of 61 patients with HCC exceeding Milan criteria and found that pretreatment AFP >1000 ng/mL was the only significant variable predicting down-staging failure (Yao et al. 2008). In a recent study by the same author including a larger cohort of 118 patients with HCC, AFP >1000 ng/mL was again found to be a significant predictor of dropout in the down-staged patient group (Yao et al. 2015).

On the other hand, a low AFP level was reported as an independent predictor of effective LRT. Bova et al. (2013) showed that an AFP level <100 ng/mL predicted successful down-staging of HCC lesions beyond Milan criteria treated with intra-arterial therapy (TACE or TARE) (Bova et al. 2013).

AFP After Down-Staging Can Predict Transplant Outcome

AFP changes after down-staging were shown by a number of studies to predict transplant outcome. As mentioned before, Merani et al. (2011) reviewed data of 6817 HCC patients from the Scientific Registry of Transplant Recipients and found that patients with AFP reduced to ≤400 ng/mL at transplantation, as a result of successful down-staging, had significantly better 3-year intent-to-treat survival than those who failed to reduce their AFP to ≤400 ng/mL (81% vs. 48%, $P \leq 0.001$). Moreover, there was no significant survival difference between patients with AFP reduced to ≤400 ng/mL and those with AFP persistently below 400 ng/mL ($P = 0.14$) (Merani et al. 2011).

As mentioned earlier, the study of Berry et al. (2013) showed the significance of AFP rather than tumor burden predicting survival after liver transplantation. An equally significant conclusion of the study was that down-staging of serum AFP was associated with down-staging of posttransplant mortality. Patients whose AFP decreased (from >320 to ≤320 or from 16–320 to 0–15 ng/mL) had posttransplant mortality similar to the group of patients with the lower AFP range. So, patients initially expected to have poor survival because of high AFP levels can still have better posttransplant outcome with down-staging if their serum AFP levels subsequently decrease (Berry and Ioannou 2013).

Similarly, but with higher AFP figures, Hameed et al. (2014) showed that patients with an initial AFP level >1000 ng/mL that subsequently decreased to <1000 ng/mL after LRT had lower recurrence risk and more favorable prognosis after liver transplantation (Hameed et al. 2014).

Since elevation of AFP is associated with higher posttransplant HCC recurrence risk that may be reduced with pretransplant LRT, AFP monitoring for HCC transplant candidates while on the waiting list has been recommended by a recent consensus conference on HCC management (Clavien et al. 2012).

AFP Monitoring After Liver Transplantation

Patients transplanted for HCC have an 8–20% risk of tumor recurrence, which usually occurs in the first 2 years after transplantation (Zimmerman et al. 2008). Few studies addressed posttransplant monitoring protocols for HCC patients. Even though AFP has no longer been part of the screening and diagnosis of HCC in most of the recent guidelines, yet its monitoring has been suggested for HCC screening after liver transplantation. The recommended protocol involves a contrast-enhanced CT or MRI every 6–12 months and AFP measurement. More frequent imaging hasn't been proven cost-effective, but it is indicated in the presence of abnormal AFP concentrations (Clavien et al. 2012).

Predictive Value of Posttransplant AFP Levels

Apart from screening for HCC recurrence, literature reporting significance of AFP monitoring after transplantation is scarce. A study from Japan highlighted factors predicting survival at the time of HCC recurrence after live donor liver transplantation in 167 recipients with HCC. AFP ≥300 ng/mL at recurrence was a significant predictor of poor prognosis among other factors, the most important of which was neutrophil/lymphocyte ratio (NLR) of ≧4 (Harimoto et al. 2013).

Other Tumor Markers for HCC

Due to the limitations of AFP in diagnosis of HCC, other tumor markers have been used alone or in combination with AFP for diagnosis and prediction of prognosis of HCC

- **AFP-L3**
  Lens culinaris agglutinin-reactive AFP (AFP-L3) is a variant of AFP that is expressed as a percentage of the total AFP. It was reported to have higher sensitivity and specificity for HCC at a cutoff >15%. However, it is of limited usefulness since it was mainly studied in Asian populations who already have elevated AFP (Kim et al. 2016).
- **Des-γ-Carboxyprothrombin (DCP)**
  Prothrombin-induced vitamin K absence-II (PIVKA-II) is an abnormal prothrombin found in the sera of HCC patients. Measured alone, it showed low sensitivity (48–62%) but good specificity for HCC (Kim et al. 2016). Studies combining measurement of both AFP and DCP reported wide ranges of sensitivity (48–94%) and specificity (53–99%), which may be higher than those for either tumor marker alone (Song et al. 2016).

  DCP has been used as a predictor of HCC recurrence after liver resection, LRT, and eventually liver transplantation. Also, it has been incorporated in different liver transplant selection criteria with either tumor morphology (Kyoto group (Fujiki et al. 2009)), AFP (the A-P level (Todo et al. 2007)), or both (Tokyo criteria (Shindoh et al. 2014)).
- **MicroRNAs**
  MicroRNAs are small non-coding RNA molecules that regulate gene expression. MicroRNAs can be released into the circulation and can be detected in body fluids. Circulating miRNAs are associated with HCC and have been studied as possible biomarkers with promising results (Borel et al. 2012). Zhou et al. conducted a study including 934 persons to study microRNA panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801). They reported a satisfactory performance of the microRNA panel in diagnosis

of HCC regardless of its stage. MicroRNA panel could differentiate HCC from healthy, chronic hepatitis B, and cirrhosis (Zhou et al. 2011).

– **Other several biomarkers** have been studied and showed promising results but still not recommended for surveillance and diagnosis of HCC. These include glypican-3 (a heparin sulfate proteoglycan), heat shock proteins, vascular endothelial growth factor, interleukin-8, transforming growth factor-beta 1, tumor-specific growth factor, and squamous cell carcinoma antigen (Kim et al. 2016).

Summary Points

- AFP is neither sensitive nor specific for HCC and is no longer used for HCC surveillance or diagnosis as per most of the recent guidelines.
- The recommended surveillance protocol for HCC is by ultrasound examination only every 6 months.
- Diagnosis of HCC is radiological by demonstration of the typical enhancement pattern of HCC on 4-phase dynamic cross-sectional imaging studies with liver mass biopsy reserved for equivocal cases.
- The staging algorithm of the Barcelona Clinic Liver Cancer (BCLC) is the most beneficial and widely used staging system.
- Liver transplantation is the gold standard treatment for selected patients with chronic liver disease and HCC.
- Milan criteria remain the gold standard liver transplant selection criteria for HCC patients.
- Higher pretransplant absolute AFP values >1000 ng/mL correlate with microvascular invasion, poor differentiation, higher posttransplant HCC recurrence, and poor survival.
- Lower AFP cutoff values were suggested by different studies, and there is no universal agreement on a specific cutoff value till now.
- A rising AFP slope of ≥ 15 ng/mL/month may be more significant than static AFP values predicting posttransplant recurrence and poor survival.
- Several transplant selection criteria were proposed incorporating AFP with different tumor parameters as the number and size, total volume, total diameter, etc. In spite of the promising results that compare to Milan, none of these criteria have been universally agreed upon or recommended by the most recent guidelines.
- The AFP model of Duvoux et al. (2012) has been validated and appears to be an accurate prognostic model compared to other criteria including Milan criteria.
- Incorporating pretransplant AFP into Milan criteria may exclude more candidates from liver transplantation, yet it may improve the performance of Milan criteria in prediction of posttransplant HCC recurrence and survival.
- LRTs can be used for "bridging" patients with T2 tumors to liver transplant if the expected waiting time on the list is ≥ 6 months.

- Dropout probability scores incorporating AFP with tumor parameters and MELD score were proposed for prioritization of HCC patients for liver transplant instead of the exception MELD points, but not yet agreed upon.
- Down-staging is a valid alternative to the controversial expansion of transplant selection criteria.
- AFP can predict the outcome of LRTs for bridging/down-staging HCC patients.
- Reduction of AFP as a result of successful down-staging predicts favorable posttransplant outcome.
- It is recommended to monitor AFP pre- and posttransplantation for HCC patients.
- High AFP at the time of diagnosis of HCC recurrence after transplantation may predict treatment failure and poor prognosis.
- Other biological markers as AFP-L3 and DCP may have a role in diagnosis of HCC.
- A number of new biomarkers and genetic markers related to HCC are being studied with promising results.

References

Adachi Y, Tsuchihashi J, Shiraishi N, Yasuda K, Etoh T, Kitano S. AFP-producing gastric carcinoma: multivariate analysis of prognostic factors in 270 patients. Oncology. 2003; 65(2):95–101.

Adinolfi A, Adinolfi M, Lessof. Alpha-feto-protein during development and in disease. J Med Genet. 1975;12(2):138–51. Pubmed Central PMCID: 1013256.

Agopian VG, Harlander-Locke M, Zarrinpar A, Kaldas FM, Farmer DG, Yersiz H, et al. A novel prognostic nomogram accurately predicts hepatocellular carcinoma recurrence after liver transplantation: analysis of 865 consecutive liver transplant recipients. J Am Coll Surg. 2015a; 220(4):416–27.

Agopian VG, Morshedi MM, McWilliams J, Harlander-Locke MP, Markovic D, Zarrinpar A, et al. Complete pathologic response to pretransplant locoregional therapy for hepatocellular carcinoma defines cancer cure after liver transplantation: analysis of 501 consecutively treated patients. Ann Surg. 2015b;262(3):536–45. discussion 43–5.

Barakat O, Wood RP, Ozaki CF, Ankoma-Sey V, Galati J, Skolkin M, et al. Morphological features of advanced hepatocellular carcinoma as a predictor of downstaging and liver transplantation: an intention-to-treat analysis. Liver Transpl. 2010;16(3):289–99.

Benson 3rd AB, D'Angelica MI, Abrams TA, Are C, Bloomston PM, Chang DT, et al. Hepatobiliary cancers, version 2.2014. J Natl Compr Canc Netw. 2014;12(8):1152–82.

Berry K, Ioannou GN. Serum alpha-fetoprotein level independently predicts posttransplant survival in patients with hepatocellular carcinoma. Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society. 2013 Jun;19(6):634-45. PubMed PMID: 23536495.

Bismuth H, Chiche L, Adam R, Castaing D, Diamond T, Dennison A. Liver resection versus transplantation for hepatocellular carcinoma in cirrhotic patients. Ann Surg. 1993; 218(2):145–51. Pubmed Central PMCID: 1242923, Epub 1993/08/01. eng.

Borel F, Konstantinova P, Jansen PL. Diagnostic and therapeutic potential of miRNA signatures in patients with hepatocellular carcinoma. J Hepatol. 2012;56(6):1371–83.

Bova V, Miraglia R, Maruzzelli L, Vizzini GB, Luca A. Predictive factors of downstaging of hepatocellular carcinoma beyond the Milan criteria treated with intra-arterial therapies. Cardiovasc Intervent Radiol. 2013;36(2):433–9.

Bruix J, Sherman M, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. Hepatology. 2011;53(3):1020–2. Pubmed Central PMCID: 3084991.

Bruix J, Han KH, Gores G, Llovet JM, Mazzaferro V. Liver cancer: approaching a personalized care. J Hepatol. 2015;62(1 Suppl):S144–56. Pubmed Central PMCID: 4520430.

Bruix J, Reig M, Sherman M. Evidence-based diagnosis, staging, and treatment of patients with hepatocellular carcinoma. Gastroenterology. 2016;150(4):835–53.

Busuttil RW. Liver transplantation for hepatocellular carcinoma: the Hangzhou experience. Hepatobiliary Pancreat Dis Int. 2008;7(3):235–6.

Cescon M, Cucchetti A, Ravaioli M, Pinna AD. Hepatocellular carcinoma locoregional therapies for patients in the waiting list. Impact on transplantability and recurrence rate. J Hepatol. 2013;58(3):609–18.

Chaiteerakij R, Zhang X, Addissie BD, Mohamed EA, Harmsen WS, Theobald PJ, et al. Combinations of biomarkers and Milan criteria for predicting hepatocellular carcinoma recurrence after liver transplantation. Liver Transpl. 2015;21(5):599–606. Pubmed Central PMCID: 4490162.

Clavien P-A, Lesurtel M, Bossuyt PMM, Gores GJ, Langer B, Perrier A. Recommendations for liver transplantation for hepatocellular carcinoma: an international consensus conference report. Lancet Oncol. 2012;13(1):e11–22.

Cucchetti A, Cescon M, Bigonzi E, Piscaglia F, Golfieri R, Ercolani G, et al. Priority of candidates with hepatocellular carcinoma awaiting liver transplantation can be reduced after successful bridge therapy. Liver Transpl. 2011a;17(11):1344–54.

Cucchetti A, Cescon M, Bertuzzo V, Bigonzi E, Ercolani G, Morelli MC, et al. Can the dropout risk of candidates with hepatocellular carcinoma predict survival after liver transplantation? Am J Transplant. 2011b;11(8):1696–704.

Deutsch HF. Chemistry and biology of alpha-fetoprotein. Adv Cancer Res. 1991;56:253–312.

DuBay DA, Sandroussi C, Kachura JR, Ho CS, Beecroft JR, Vollmer CM, et al. Radiofrequency ablation of hepatocellular carcinoma as a bridge to liver transplantation. HPB. 2011; 13(1):24–32. Pubmed Central PMCID: 3019538.

Duffy JP, Vardanian A, Benjamin E, Watson M, Farmer DG, Ghobrial RM, et al. Liver transplantation criteria for hepatocellular carcinoma should be expanded: a 22-year experience with 467 patients at UCLA. Ann Surg. 2007;246(3):502–9. Pubmed Central PMCID: 1959350, discussion 9–11.

Dumitra TC, Dumitra S, Metrakos PP, Barkun JS, Chaudhury P, Deschenes M, et al. Pretransplantation alpha-fetoprotein slope and Milan criteria: strong predictors of hepatocellular carcinoma recurrence after transplantation. Transplantation. 2013;95(1):228–33.

Duvoux C, Roudot-Thoraval F, Decaens T, Pessione F, Badran H, Piardi T, et al. Liver transplantation for hepatocellular carcinoma: a model including alpha-fetoprotein improves the performance of Milan criteria. Gastroenterology. 2012;143(4):986–94.e3; quiz e14-5.

El-Serag HB, Kanwal F. Epidemiology of hepatocellular carcinoma in the United States: where are we? Where do we go? Hepatology. 2014;60(5):1767–75. Pubmed Central PMCID: 4211957.

European Association for the Study of the Liver, European Organisation for Research and Treatment of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol. 2012;56(4):908–43.

Frangakis C, Geschwind JF, Kim D, Chen Y, Koteish A, Hong K, et al. Chemoembolization decreases drop-off risk of hepatocellular carcinoma patients on the liver transplant list. Cardiovasc Intervent Radiol. 2011;34(6):1254–61. Pubmed Central PMCID: 4137764.

Freeman RB, Edwards EB, Harper AM. Waiting list removal rates among patients with chronic and malignant liver diseases. Am J Transplant. 2006;6(6):1416–21.

Fujiki M, Takada Y, Ogura Y, Oike F, Kaido T, Teramukai S, et al. Significance of des-gamma-carboxy prothrombin in selection criteria for living donor liver transplantation for hepatocellular carcinoma. Am J Transplant. 2009;9(10):2362–71.

Galuppo R, McCall A, Gedaly R. The role of bridging therapy in hepatocellular carcinoma. Int J Hepatol. 2013;2013:419302. Pubmed Central PMCID: 3880689.

Gebo KA, Chander G, Jenckes MW, Ghanem KG, Herlong HF, Torbenson MS, et al. Screening tests for hepatocellular carcinoma in patients with chronic hepatitis C: a systematic review. Hepatology. 2002;36(5 Suppl 1):S84–92.

Gitlin D, Perricelli A, Gitlin GM. Synthesis of -fetoprotein by liver, yolk sac, and gastrointestinal tract of the human conceptus. Cancer Res. 1972;32(5):979–82.

Golfieri R, Coppola F, Fusco F, Li Bassi S, Caraceni P, Bernardi M, et al. Malignant progression of a small HCC nodule: hypovascular "early HCC" converted to hypervascular "small HCC" within six months. Dig Liver Dis. 2007;39(9):883–90.

Gordon-Weeks AN, Snaith A, Petrinic T, Friend PJ, Burls A, Silva MA. Systematic review of outcome of downstaging hepatocellular cancer before liver transplantation in patients outside the Milan criteria. Br J Surg. 2011;98(9):1201–8.

Gouw AS, Balabaud C, Kusano H, Todo S, Ichida T, Kojiro M. Markers for microvascular invasion in hepatocellular carcinoma: where do we stand? Liver Transpl. 2011;17 Suppl 2:S72–80.

Hakeem AR, Young RS, Marangoni G, Lodge JP, Prasad KR. Systematic review: the prognostic role of alpha-fetoprotein following liver transplantation for hepatocellular carcinoma. Aliment Pharmacol Ther. 2012;35(9):987–99.

Hameed B, Mehta N, Sapisochin G, Roberts JP, Yao FY. Alpha-fetoprotein level $>$ 1000 ng/mL as an exclusion criterion for liver transplantation in patients with hepatocellular carcinoma meeting the Milan criteria. Liver Transpl. 2014;20(8):945–51. Pubmed Central PMCID: 4807739.

Han K, Tzimas GN, Barkun JS, Metrakos P, Tchervenkov JL, Hilzenrat N, et al. Preoperative alpha-fetoprotein slope is predictive of hepatocellular carcinoma recurrence after liver transplantation. J Can Gastroenterol. 2007;21(1):39–45. Pubmed Central PMCID: 2656629.

Harimoto N, Shirabe K, Nakagawara H, Toshima T, Yamashita Y, Ikegami T, et al. Prognostic factors affecting survival at recurrence of hepatocellular carcinoma after living-donor liver transplantation: with special reference to neutrophil/lymphocyte ratio. Transplantation. 2013;96(11):1008–12.

Harper ME, Dugaiczyk A. Linkage of the evolutionarily-related serum albumin and alpha-fetoprotein genes within q11-22 of human chromosome 4. Am J Hum Genet. 1983;35 (4):565–72. Pubmed Central PMCID: 1685723.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69–90.

Jonas S, Bechstein WO, Steinmuller T, Herrmann M, Radke C, Berg T, et al. Vascular invasion and histopathologic grading determine outcome after liver transplantation for hepatocellular carcinoma in cirrhosis. Hepatology. 2001;33(5):1080–6.

Kashkoush S, El Moghazy W, Kawahara T, Gala-Lopez B, Toso C, Kneteman NM. Three-dimensional tumor volume and serum alpha-fetoprotein are predictors of hepatocellular carcinoma recurrence after liver transplantation: refined selection criteria. Clin Transplant. 2014;28(6):728–36.

Kim JU, Shariff MI, Crossey MM, Gomez-Romero M, Holmes E, Cox IJ, et al. Hepatocellular carcinoma: review of disease and tumor biomarkers. World J Hepatol. 2016;8(10):471–84. Pubmed Central PMCID: 4820639.

Lai Q, Melandro F, Pinheiro RS, Donfrancesco A, Fadel BA, Levi Sandri GB, et al. Alpha-fetoprotein and novel tumor biomarkers as predictors of hepatocellular carcinoma recurrence after surgery: a brilliant star raises again. Int J Hepatol. 2012a;2012:893103. Pubmed Central PMCID: 3391901.

Lai Q, Avolio AW, Manzia TM, Sorge R, Agnes S, Tisone G, et al. Combination of biological and morphological parameters for the selection of patients with hepatocellular carcinoma waiting for liver transplantation. Clin Transplant. 2012b;26(2):E125–31.

Lai Q, Avolio AW, Graziadei I, Otto G, Rossi M, Tisone G, et al. Alpha-fetoprotein and modified response evaluation criteria in solid tumors progression after locoregional therapy as predictors of hepatocellular cancer recurrence and death after transplantation. Liver Transpl. 2013;19 (10):1108–18.

Lai Q, Inostroza M, Rico Juri JM, Goffette P, Lerut J. Delta-slope of alpha-fetoprotein improves the ability to select liver transplant patients with hepatocellular cancer. HPB. 2015;17(12):1085–95. Pubmed Central PMCID: 4644360.

Lao OB, Weissman J, Perkins JD. Pre-transplant therapy for hepatocellular carcinoma is associated with a lower recurrence after liver transplantation. Clin Transplant. 2009;23(6):874–81.

Llovet JM, Fuster J, Bruix J. Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: resection versus transplantation. Hepatology. 1999;30(6):1434–40.

Lok AS, Sterling RK, Everhart JE, Wright EC, Hoefs JC, Di Bisceglie AM, et al. Des-gamma-carboxy prothrombin and alpha-fetoprotein as biomarkers for the early detection of hepatocellular carcinoma. Gastroenterology. 2010;138(2):493–502. Pubmed Central PMCID: 2819612.

Mailey B, Artinyan A, Khalili J, Denitz J, Sanchez-Luege N, Sun CL, et al. Evaluation of absolute serum alpha-fetoprotein levels in liver transplant for hepatocellular cancer. Arch Surg. 2011;146 (1):26–33.

Majno P, Lencioni R, Mornex F, Girard N, Poon RT, Cherqui D. Is the treatment of hepatocellular carcinoma on the waiting list necessary? Liver Transpl. 2011;17 Suppl 2:S98–108.

Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med. 1996;334(11):693–9. Epub 1996/03/14. eng.

Mazzaferro V, Llovet JM, Miceli R, Bhoori S, Schiavo M, Mariani L, et al. Predicting survival after liver transplantation in patients with hepatocellular carcinoma beyond the Milan criteria: a retrospective, exploratory analysis. Lancet Oncol. 2009;10(1):35–43. Epub 2008/12/09. eng.

Mazzaferro V, Bhoori S, Sposito C, Bongini M, Langer M, Miceli R, et al. Milan criteria in liver transplantation for hepatocellular carcinoma: an evidence-based analysis of 15 years of experience. Liver Transpl. 2011;17 Suppl 2:S44–57. Epub 2011/06/23. eng.

Mehta N, Dodge JL, Goel A, Roberts JP, Hirose R, Yao FY. Identification of liver transplant candidates with hepatocellular carcinoma and a very low dropout risk: implications for the current organ allocation policy. Liver Transpl. 2013;19(12):1343–53. Pubmed Central PMCID: 3883622.

Merani S, Majno P, Kneteman NM, Berney T, Morel P, Mentha G, et al. The impact of waiting list alpha-fetoprotein changes on the outcome of liver transplant for hepatocellular carcinoma. J Hepatol. 2011;55(4):814–9.

Moya A, Berenguer M, Aguilera V, Juan FS, Nicolas D, Pastor M, et al. Hepatocellular carcinoma: can it be considered a controversial indication for liver transplantation in centers with high rates of hepatitis C? Liver Transpl. 2002;8(11):1020–7.

Nikolic JA. Synthesis, structure and function of alpha-fetoproteins and their importance in medicine. Glas Srp Akad Nauka Med. 1992;42:57–73. Sinteza, struktura i funkcija alfa-fetoproteina i njegov znacaj u medicini.

Otto G, Herber S, Heise M, Lohse AW, Monch C, Bittinger F, et al. Response to transarterial chemoembolization as a biological selection criterion for liver transplantation in hepatocellular carcinoma. Liver Transpl. 2006;12(8):1260–7.

Otto G, Schuchmann M, Hoppe-Lotichius M, Heise M, Weinmann A, Hansen T, et al. How to decide about liver transplantation in patients with hepatocellular carcinoma: size and number of lesions or response to TACE? J Hepatol. 2013;59(2):279–84.

Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol. 2006;45(4):529–38.

Pomfret EA, Washburn K, Wald C, Nalesnik MA, Douglas D, Russo M, et al. Report of a national conference on liver allocation in patients with hepatocellular carcinoma in the United States. Liver Transpl. 2010;16(3):262–78.

Pompili M, Francica G, Ponziani FR, Iezzi R, Avolio AW. Bridging and downstaging treatments for hepatocellular carcinoma in patients on the waiting list for liver transplantation. World J Gastroenterol. 2013;19(43):7515–30. Pubmed Central PMCID: 3837250.

Riaz A, Ryu RK, Kulik LM, Mulcahy MF, Lewandowski RJ, Minocha J, et al. Alpha-fetoprotein response after locoregional therapy for hepatocellular carcinoma: oncologic marker of radiologic response, progression, and survival. J Clin Oncol. 2009;27(34):5734–42.

Sato Y, Sekine T, Ohwada S. Alpha-fetoprotein-producing rectal cancer: calculated tumor marker doubling time. J Surg Oncol. 1994;55(4):265–8.

Shetty K, Timmins K, Brensinger C, Furth EE, Rattan S, Sun W, et al. Liver transplantation for hepatocellular carcinoma validation of present selection criteria in predicting outcome. Liver Transpl. 2004;10(7):911–8.

Shindoh J, Sugawara Y, Nagata R, Kaneko J, Tamura S, Aoki T, et al. Evaluation methods for pretransplant oncologic markers and their prognostic impacts in patient undergoing living donor liver transplantation for hepatocellular carcinoma. Transpl Int. 2014;27(4):391–8.

Singal A, Volk ML, Waljee A, Salgia R, Higgins P, Rogers MA, et al. Meta-analysis: surveillance with ultrasound for early-stage hepatocellular carcinoma in patients with cirrhosis. Aliment Pharmacol Ther. 2009;30(1):37–47.

Song P, Feng X, Inagaki Y, Song T, Zhang K, Wang Z, et al. Clinical utility of simultaneous measurement of alpha-fetoprotein and des-γ-carboxy prothrombin for diagnosis of patients with hepatocellular carcinoma in China: a multi-center case-controlled study of 1,153 subjects. BioSci Trends. 2014;8(5):266–73.

Song PP, Xia JF, Inagaki Y, Hasegawa K, Sakamoto Y, Kokudo N, et al. Controversies regarding and perspectives on clinical utility of biomarkers in hepatocellular carcinoma. World J Gastroenterol. 2016;22(1):262–74. Pubmed Central PMCID: 4698491.

Sterling RK, Wright EC, Morgan TR, Seeff LB, Hoefs JC, Di Bisceglie AM, et al. Frequency of elevated hepatocellular carcinoma (HCC) biomarkers in patients with advanced hepatitis C. Am J Gastroenterol. 2012;107(1):64–74. Pubmed Central PMCID: 3903319.

Takamori R, Wong LL, Dang C, Wong L. Needle-tract implantation from hepatocellular cancer: is needle biopsy of the liver always necessary? Liver Transpl. 2000;6(1):67–72.

Tateishi R, Yoshida H, Matsuyama Y, Mine N, Kondo Y, Omata M. Diagnostic accuracy of tumor markers for hepatocellular carcinoma: a systematic review. Hepatol Int. 2008;2(1):17–30. Pubmed Central PMCID: 2716871.

Todo S, Furukawa H, Tada M, Japanese Liver Transplantation Study Group. Extending indication: role of living donor liver transplantation for hepatocellular carcinoma. Liver Transpl. 2007;13 (11 Suppl 2):S48–54.

Toso C, Asthana S, Bigam DL, Shapiro AM, Kneteman NM. Reassessing selection criteria prior to liver transplantation for hepatocellular carcinoma utilizing the Scientific Registry of Transplant Recipients database. Hepatology. 2009;49(3):832–8.

Toso C, Mentha G, Kneteman NM, Majno P. The place of downstaging for hepatocellular carcinoma. J Hepatol. 2010;52(6):930–6.

Toso C, Dupuis-Lozeron E, Majno P, Berney T, Kneteman NM, Perneger T, et al. A model for dropout assessment of candidates with or without hepatocellular carcinoma on a common liver transplant waiting list. Hepatology. 2012;56(1):149–56.

Toso C, Majno P, Berney T, Morel P, Mentha G, Combescure C. Validation of a dropout assessment model of candidates with/without hepatocellular carcinoma on a common liver transplant waiting list. Transpl Int. 2014;27(7):686–95.

Varona MA, Soriano A, Aguirre-Jaime A, Garrido S, Oton E, Diaz D, et al. Risk factors of hepatocellular carcinoma recurrence after liver transplantation: accuracy of the alpha-fetoprotein model in a single-center experience. Transplant Proc. 2015;47(1):84–9.

Vibert E, Azoulay D, Hoti E, Iacopinelli S, Samuel D, Salloum C, et al. Progression of alphafetoprotein before liver transplantation for hepatocellular carcinoma in cirrhotic patients: a critical factor. Am J Transplant. 2010;10(1):129–37.

Washburn K, Edwards E, Harper A, Freeman R. Hepatocellular carcinoma patients are advantaged in the current liver transplant allocation system. Am J Transplant. 2010;10(7):1643–8.

Xu X, Lu D, Ling Q, Wei X, Wu J, Zhou L, et al. Liver transplantation for hepatocellular carcinoma beyond the Milan criteria. Gut 2015;0: 1–7.

Yamashiki N, Gaynor JJ, Kato T, Reddy KR, Sobhonslidsuk A, Levi D, et al. Competing risks analysis of predictors of delisting owing to tumor progression in liver transplant candidates with hepatocellular carcinoma. Am J Transplant. 2004;4(5):774–81.

Yang SH, Suh KS, Lee HW, Cho EH, Cho JY, Cho YB, et al. A revised scoring system utilizing serum alphafetoprotein levels to expand candidates for living donor transplantation in hepatocellular carcinoma. Surgery. 2007;141(5):598–609.

Yao FY, Ferrell L, Bass NM, Watson JJ, Bacchetti P, Venook A, et al. Liver transplantation for hepatocellular carcinoma: expansion of the tumor size limits does not adversely impact survival. Hepatology. 2001;33(6):1394–403.

Yao FY, Xiao L, Bass NM, Kerlan R, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: validation of the UCSF-expanded criteria based on preoperative imaging. Am J Transplant. 2007;7(11):2587–96.

Yao FY, Kerlan Jr RK, Hirose R, Davern 3rd TJ, Bass NM, Feng S, et al. Excellent outcome following down-staging of hepatocellular carcinoma prior to liver transplantation: an intention-to-treat analysis. Hepatology. 2008;48(3):819–27. Pubmed Central PMCID: 4142499.

Yao FY, Mehta N, Flemming J, Dodge J, Hameed B, Fix O, et al. Downstaging of hepatocellular cancer before liver transplant: long-term outcome compared to tumors within Milan criteria. Hepatology. 2015;61(6):1968–77. Pubmed Central PMCID: 4809192.

Zheng SS, Xu X, Wu J, Chen J, Wang WL, Zhang M, et al. Liver transplantation for hepatocellular carcinoma: Hangzhou experiences. Transplantation. 2008;85(12):1726–32.

Zhou J, Yu L, Gao X, Hu J, Wang J, Dai Z, et al. Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. J Clin Oncol. 2011;29(36):4781–8.

Zimmerman MA, Ghobrial RM, Tong MJ, Hiatt JR, Cameron AM, Hong J, et al. Recurrence of hepatocellular carcinoma following liver transplantation: a review of preoperative and postoperative prognostic indicators. Arch Surg. 2008;143(2):182–8. discussion 8.

Zou WL, Zang YJ, Chen XG, Shen ZY. Risk factors for fatal recurrence of hepatocellular carcinoma and their role in selecting candidates for liver transplantation. Hepatobiliary Pancreat Dis Int. 2008;7(2):145–51.

Immunological Biomarkers in Liver Transplantation

41

Estela Solanas, Elena Martínez-Crespo, Alberto Lue, Pedro Baptista, and M. Trinidad Serrano

Contents

Abstract

Liver transplantation is the standard therapy for many liver diseases. Despite being a considerable successful treatment, avoiding allograft rejection, among other complications, continues being one of the big challenges for physicians. Immunosuppression drugs significantly decrease rejection rates after liver transplantation; however, they have generally associated adverse effects which compromise liver transplantation outcome, increasing patients' morbidity and

E. Solanas (✉) • P. Baptista
Digestive Pathology Group, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain
e-mail: emsolanas@iisaragon.es; pbaptista.iacs@aragon.es

E. Martínez-Crespo • A. Lue • M.T. Serrano (✉)
Liver Transplantation Unit, Gastroenterology and Hepatology Department, University Hospital Lozano Blesa, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain
e-mail: emartinezcr@salud.aragon.es; alberto.lue@hotmail.com; tserrano.aullo@gmail.com; utra.hcu@salud.aragon.com

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_17

mortality. So, a close monitoring of immunosuppression is essential to reduce drugs' undesirable effects as long as allograft rejection is avoided. Nevertheless, monitoring of liver transplant recipients (LTRs) frequently entails the study of liver biopsies with its consequent inconvenience and risk for the patient. Identification of biomarkers that could diagnose or predict the risk of suffering allograft rejection (acute, chronic, or antibody mediated), or, on the contrary, the potential to achieve allograft tolerance, would represent a considerable progress in the managing and monitoring of LTRs. As the immune response of LTRs is responsible for the rejection or tolerance of the liver allograft, most of the potential biomarkers studied in this field are related to the immune system. For that reason, in this chapter, we attempt to review the state of the art in immunological biomarkers for the managing of patients after liver transplantation.

Keywords

Liver transplantation • Immunology • Allograft tolerance • Rejection • Biological markers • Immunologic monitoring

List of Abbreviations

ALT	Alanine transaminase
AMR	Antibody-mediated rejection
APCs	Antigen-presenting cells
AR	Acute rejection
BEC	Biliary epithelial cells
C1q	Complement component 1q
C4	Complement component 4
CLU	Clusterin
CMV	Cytomegalovirus
CR	Chronic rejection
DC	Dendritic cells
DSAs	Donor-specific human alloantibodies
GBP2	Guanylate-binding protein 2
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
HLA-G	Human leukocyte antigen, class I, G
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-23	Interleukin 23
IRF5	Interferon regulatory factor 5
IS	Immunosuppression)
Krt19	Cytokeratin-19

LCN2	Lipocalin-2
LTRs	Liver transplant recipients
MCS	Median channel shift
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
miRNA	microRNA
NK	Natural killer cells
OLT	Orthotopic liver transplantation
PBMC	Peripheral blood mononuclear cell
RAI	Rejection activity index
sIL2-R	Soluble interleukin 2 receptor
SPIs	Solid-phase immunoassays
Th1	Type 1 helper T cells
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-alpha
Treg cells	Regulatory T cells
VCAM-1	Vascular cell adhesion protein 1

Key Facts

Key Facts of Acute Rejection (AR)

- AR is a T-cell-dependent immune response directed against donor tissues resulting from the recognition of alloantigens by recipient T cells.
- AR after liver transplantation occurs in as much as 70% of patients within the first year.
- Approximately 5–10% of liver transplant recipients who develop AR progress to severe ductopenic rejection despite antirejection therapy.
- AR is generally suspected based upon the development of hepatic biochemical test abnormalities although histological study of liver biopsy is required to establish the diagnosis.

Key Facts of Chronic Rejection (CR)

- CR can be broadly defined as a largely indolent but progressive form of allograft injury characterized histopathologically by two main features: severe damage and loss of small bile ducts and obliterative arteriopathy.
- In comparison to acute rejection, CR is a more indolent but more progressive form of allograft injury, which is largely irreversible and eventually results in allograft failure.
- Many cases of CR clearly evolve from severe or inadequately controlled AR episodes.
- Often the only reliable early indicator of CR is persistent and preferential elevation of γ-glutamyl transpeptidase and alkaline phosphatase, which is related to bile duct damage and loss.
- The gold standard of CR diagnosis is the histopathological study of liver biopsy.

Key Facts of Graft Tolerance

- Life-long immunosuppression regimens are still required in transplant recipients, and these represent the standard treatment in daily practice despite their many side effects that increase morbidity and mortality.
- Liver is considered as an immunologically privileged organ by its lowest incidence of rejection than other solid organ transplants.
- Around 26% of adult liver transplant recipients may stop treatment without compromising the viability of the graft, and this phenomenon is known as spontaneous operational tolerance.
- Pediatric population appears to develop tolerance more easily than adult recipients.
- Whereas B cells participate in the maintenance of tolerance in other solid organ transplantations, like kidney, NK and T cells play an essential role in the development of tolerance in liver transplantation.

Definitions of Words and Terms

Acute allograft rejection	Allograft injury produced by the setting up of the cellular immunity of the recipient, usually known as acute rejection
Allograft	Whole or part of an organ or tissue that is transplanted from one individual to another of the same species with a different genotype, which generates an inmmunological response in the recipient
Allograft tolerance	Partial or complete acceptance of the allograft by the immune system of the recipient, which allows the immunosuppression withdrawal
Antibody-mediated rejection	Acute or chronic rejection of the allograft owing to the presence of DSAs, which produces an immune response against the allograft in the recipient
Chronic allograft rejection	Allograft injury produced by successive episodes of acute rejection
Cytokines	Broad and loose category of small proteins that are important in cell signaling during the immune response
Donor-specific antibodies (DSAs)	Antibodies in the allograft recipient against specific HLA donor allograft antigens, formed in the recipient prior or after transplant
Immunological markers	Biomarkers related to the allograft recipient immune system

Immunosuppression	Inhibition of immune response in the allograft recipient in order to decrease allograft injury produced by the recipient immune system
Orthotopic liver transplantation	Replacement of a diseased liver with part or whole of a healthy liver from another person (allograft) in the same anatomic location as the original liver

Introduction

Liver transplantation is the standard therapy for many liver diseases. It is indicated for severe acute or chronic liver disease where the limits of medical therapy have been reached. Successful liver transplantation results in prolonged survival and improves quality of life of recipients. Nowadays in Europe the 5-year survival rate of liver transplantation recipients is around 75%, and the long-term management of these patients is a defiant challenge for the physicians (Adam et al. 2012).

Rejection was the biggest limitation to an acceptable survival in the beginning of liver transplantation era. The development of powerful immunosuppressant agents drove to a dramatical improvement in the recipients' survival (Dienstag and Cosimi 2012).

Immunosuppression drugs significantly decrease rejection rates after liver transplantation. However, these agents have a poor safety profile, and in some cases, they are not well tolerated and require a close monitoring to prevent toxicity. Adverse effects associated with immunosuppressant therapy after liver transplantation include neurotoxicity, renal function impairment, increased risk of de novo cancer, or increased cardiovascular risk (Adams et al. 2015). These comorbidities are the main reason that transplant recipients still exhibit much higher morbidity and mortality than the general population (Londoño et al. 2012).

Lowering the dose or changing the immunosuppressant agent is frequent after liver transplantation to prevent or control adverse effects and to ease the development of acute or chronic rejection. Current immunological monitoring after orthotopic liver transplantation (OLT) relies mainly on clinical judgment and on measurement of immunosuppressive drug levels, without a real assessment of the immunological system suppression. Therefore, the evaluation of the immunosuppression state in liver transplanted patients is crucial for a correct posttransplant management and constitutes a major step toward the personalization of immunosuppressive therapy (Adams et al. 2015). The availability of biomarkers to identify patients in high risk of acute rejection (AR) could help to identify subjects that need an aggressive immunosuppressant therapy in the early posttransplant period. On the contrary, a biomarker to predict graft acceptance could allow using low dose of immunosuppressant drugs that could avoid or decrease the rate of adverse effects.

Despite the elevated interest in the evaluation of potential biomarkers of AR and graft acceptance, only a few of them are used routinely in the clinical practice.

Several biomarkers have been evaluated in the set of AR. Substances that increase during AR, such as liver enzymes and pro-inflammatory cytokines, have been the most studied ones. However most of them do not allow to differentiate AR from other OLT complications, as infections (Germani et al. 2015).

In the evaluation of graft acceptance, the results are more encouraging. Liver biopsy is the gold standard to assess graft status after liver transplantation, but is an invasive procedure, and it does not permit to identify the tolerant recipients (Germani et al. 2015). Several studies have been performed to identify biomarkers of tolerance after liver transplant. Patients undergoing immunosuppression withdrawal seem to present specific characteristics compared to non-tolerant patients. Most of them are based on the immunophenotyping of peripheral blood samples and nonspecific genome analysis (Londoño et al. 2012).

In this chapter, we attempt to review the state of the art of immunological biomarkers in liver transplantation.

Immunological Basis of Allograft Rejection in Liver Transplantation

Generally, liver allograft rejection involves predominantly graft-versus-host reactions after transplantation. The lymphocyte-mediated reactions from the recipient to the allogeneic cells (acquired as a graft) lead to injury and/or the destruction of the grafted cells. According with the time it takes to occur and the implicated causes, graft rejection has been divided into four groups (Table 1).

However, mechanisms that are at the genesis of rejection all are put in motion with transplantation of an allogeneic graft (Fig. 1). Following transplantation of liver or other solid organs, antibody-mediated hyperacute vasculitic rejection can develop in individuals with preformed antibodies targeting the donor's major histocompatibility complex (MHC) class I-encoded antigens. However, in liver allograft transplant, owing to the liver tolerogenic capacity, hyperacute rejection is very unusual (Adams et al. 2015).

Under most other circumstances, AR is initiated by the large number of the host's T cells that recognize donor alloantigens (Afzali et al. 2008). Transplantation of MHC histo-incompatible organs and tissues therefore produces a strong, cytopathic T-cell-dependent immune response to donor tissues. In the direct pathway (dominant in AR), allogeneic MHC molecules on donor antigen-presenting cells (APCs) are recognized by recipient T cells without any previous processing. The initial T response in AR is characterized by infiltration of the graft by CD4+ and CD8+ T cells, but myeloid and innate lymphoid cells are involved and determine the outcome of the allorecognition.

Despite routine use of immunosuppressive therapy, AR is not uncommon. CD4+ and CD8+ T cells together contribute to AR, although CD4+ T cells primarily mediate the rejection response. Activation of CD4+ T cells is strongly influenced

Table 1 Liver graft rejection types and their major causes

Type of rejection	Time after OLT[a]	Cause
Hyperacute	Minutes–hours	Preformed anti-donor antibodies and complement activation
Acute	Days–months	T-cell activation
Chronic	Months–years	Unclear mechanisms
Antibody mediated	Minutes–years	Preformed or de novo anti-donor antibodies. Unclear mechanisms

Rejection types that can occur after orthotopic liver transplantation (OLT) are presented
[a]Time after OLT: posttransplantation period in which rejection symptoms start to appear

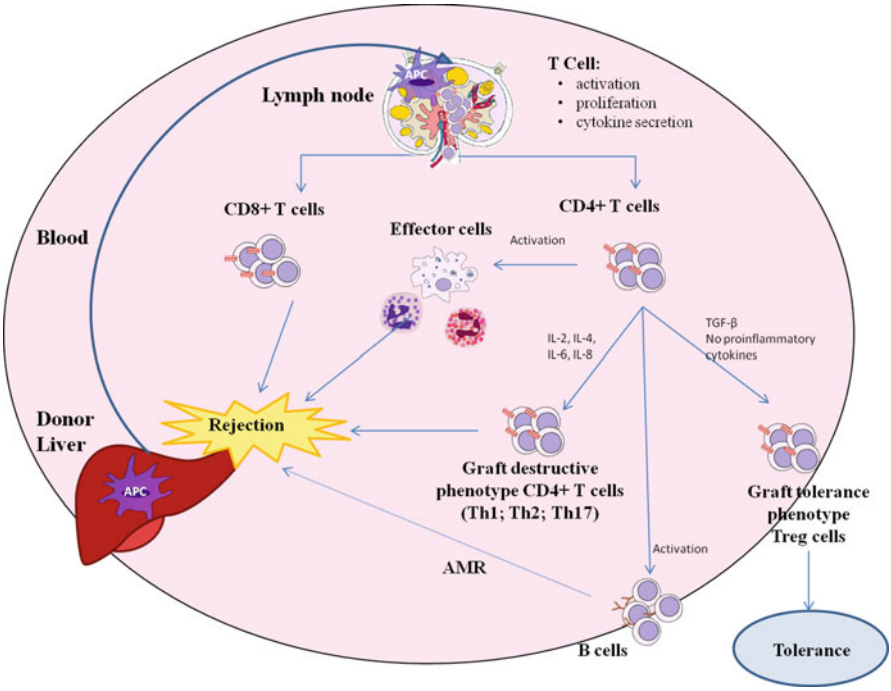

Fig. 1 Mechanisms underlying liver allograft rejection. After liver transplant, recipient T cells are activated directly (acute rejection) or indirectly (chronic rejection), and proliferation of T cells and activation of effector cells by CD4+ T cells occur. CD8+ T cells and effector cells infiltrate and injure the graft developing rejection signs. Depending on the cytokine environment, activated CD4+ T cells can transform into graft-destructive or graft-tolerance phenotypes which balance the immunological response. CD4+T cells can also activate B cells, which can develop a further antibody-mediated response against antigens in the graft and generate antibody-mediated rejection (*AMR*)

by the cytokine environment, and this contribute to the balance between rejection and tolerance, since CD4T cells can differentiate into effector (cells responsible of allograft rejection) or regulatory (cells responsible of allograft tolerance) phenotypes depending on the cytokines present during activation (Adams et al. 2015). Despite the importance of CD4+ T cells in rejection, many activated CD8+ (cytotoxic) T cells penetrate the transplant at the time of rejection, along with other mononuclear leukocytes. However, their exact role is still not completely clear. The hepatic sinusoidal endothelium presents some vascular adhesion proteins (VCAM-1, vascular cell adhesion protein 1), activated by injury or local inflammation, and mediates lymphocyte recruitment to the liver during graft rejection. Some chemokines are also critical for leukocyte recruitment (CXCL9 or CXCL10) and play an important role for compartmentalization of infiltrating leukocytes during graft rejection. Cytotoxic T cells and other effector leukocytes bind to bile ducts and hepatocytes by several molecular mechanisms causing hepatocyte and bile duct destruction during rejection.

Activated T cells also provide help for alloantibody production by B cells leading to antibody-mediated graft damage and complement deposition on graft endothelium (Adams et al. 2015).

Indirect antigen presentation (indirect pathway), in which donor alloantigens are processed by APCs, which internalize and process donor MHC molecules and subsequently present them to the recipient's T cells (Afzali et al. 2008), dominates in chronic rejection (CR) and later immune response in the graft.

Immunological Biomarkers of Rejection

Biomarkers of Acute Rejection

Acute allograft rejection is a graft damage arising as a consequence of an immunological reaction to foreign antigens on the graft. The incidence of clinically significant rejection is 10–40% in most series. The majority of first acute rejection episodes are diagnosed in the first year after transplantation, usually within the first month after transplantation, although late acute rejection can occur more than 3 months after transplantation, normally as a consequence of inadequate immunosuppression (Thurairajah et al. 2013).

Clinical suspicion on one hand, based on nonspecific symptoms, as malaise, fever, abdominal pain, hepatomegaly, and increasing ascites, and biochemical alterations on the other hand, such as elevation of serum aminotransferases, alkaline phosphatases, g-glutamyl transferases, and bilirubin levels, can make suspect of AR after OLT. However, because of the nonspecificity of these signs and symptoms that do not correlate with the severity of rejection (Abraham and Furth 1995), anatomical pathology confirmation is required on liver biopsy, which results costly and may imply complications, as bleeding, infections, and so on.

Noninvasive diagnostic tools, as biomarkers, for the early diagnosis of AR would be very valuable. Considering that AR is a consequence of the recipient immune

response, biomarkers related to this immune reaction have been studied in the last years; however, few of them have been validated and are used routinely in the clinical practice.

Serum Immunological Markers

The first potential immunological biomarkers being studied were cytokines and other proteins related to the inflammatory response.

The type 1 helper T cells (TH1) and cytokine interleukin 2 (IL-2) and its receptors (IL2-R) are well known to trigger acute allograft rejection. Perkins et al. (1989) studying 82 liver transplant recipients (LTRs) found that soluble IL2-R (sIL2-R) increased 17% per day in the 10 days prior to the diagnosis of AR episodes compared to the control group. Although sIL2-R also tended to increase in recipients developing cytomegalovirus (CMV) disease prior to the diagnosis, the increase was not as high as in the recipients with rejection. Even though other authors have found a raise in serum sIL2-R levels before diagnosis of AR, they have also observed higher serum sIL2-R levels in infection episodes after transplantation (Platz et al. 1997). In a study with 81 patients, Platz et al. (1997) found that sIL-2R increased 3 days prior to the onset of acute steroid resistant rejection. In this case, the increase in sIL-2R was similar to that in patients with serious infections and asymptomatic cholangitis. However, Ninova et al. (1994), in order to differentiate AR from CMV hepatitis after OLT, observed that patients with CMV showed a higher increased of sIL-2R and concomitant elevation of CD8+ T cells, not observed in AR patients.

Boleslawski et al. (2004) associated intracellular IL-2 expression in CD8+ T cells before transplantation with the later development of AR. These data were later confirmed by Akoglu et al. (2009), who observed that the percentage of CD8+ T cells with detectable intracellular IL-2 was significantly increased in patients with AR compared to recipients without rejection. Moreover, these authors found a good correlation between intracellular IL-2 and rejection severity, according to the Banff score (Spearmans-rho $= 0.81$, $P < 0.05$), showing good sensitivity and excellent specificity in AR (specificity of 95% for histologically proven AR). In a later study (Millán et al. 2013), LTRs who developed AR showed a significant increase in the percentage of CD8+IL-2+ T-cell levels during the early posttransplantation period (when there is a high incidence of AR) associated with lower susceptibility to immunosuppressive treatment. This increase was also accompanied by an increase in the percentage of interferon gamma (IFN-γ)-positive CD8+ T cells and soluble IFN-γ levels. The percentage of IFN-γ CD8+ T cells prior to transplantation was also higher in rejectors, and levels of soluble IFN-γ were also associated with severity of AR. Authors of this study suggested IFN-γ as a robust candidate biomarker of liver transplantation.

Other cytokines and growth factors have also been shown to increase in AR. In the study of Platz et al. (1997), mentioned before, also an elevation of tumor necrosis factor (TNF) receptor II (released upon stimulation of Th1 lymphocytes), interleukin-8 (IL-8), and interleukin-10 (IL-10) occurred in patients with steroid-resistant rejection 3 days prior to the onset of rejection; however, as in the case of

sIL-2R, similar increases were also observed in patients prior to severe infection. Neopterin (produced by IFN-γ-activated macrophages) levels increased before acute steroid-resistant rejection, although this increase was significantly lower than that observed prior to severe infection. In fact, a multivariate analysis revealed significant differences in cytokine pattern between rejection and infection for neopterin, IL-8, sTNF-RII, and IL-10, which may guide monitoring after OLT. This study also analyzed other cytokines including IFN-γ, interleukin-1-beta (IL-1β), interleukin 4 (IL-4), and interleukin 6 (IL-6), which also increased during severe rejection and infection, but, after the onset of other events, makes them of less clinical value in terms of early biomarkers.

Despite the results of Platz et al. (1997) measuring IL-6 levels in serum, Kita et al. (1994) found significantly higher serum levels of IL-6 up to 4 days prior to histopathological diagnosis of AR, and although patients with serious infections also presented higher levels of IL-6, the increase was distinguishable between both episodes.

Interleukin 15 (IL-15), produced by non-lymphatic cells as macrophages, with similar action to IL-2 and inducing proliferation of natural killer (NK) cells, increased in plasma during AR, especially in steroid-resistant rejection (Conti et al. 2003). Interleukin 23 (IL-23) and interleukin 17 (IL-17), produced by helper T cells and induced by IL-23, have been showed to be involved in the AR process after OLT. Although IL-23 and IL-17 serum levels are not different in the early posttransplantation period, they have been showed to increase at the diagnosis of AR (Fábrega et al. 2009). A later prospective study confirmed that levels of circulating CD4+IL-17+ T (Th17 cells) were higher during AR compared with LTRs without AR. This increase in the frequency of CD4+IL-17+ cells in peripheral blood was positively correlated with the rejection activity index (RAI) ($r = 0.79$, $P = 0.0002$) (Fan et al. 2012).

Apart from soluble cytokines, membrane proteins expressed on cells of the immune system have also been studied as potential markers of AR. Expression of CD28, protein expressed on the membrane of T cells that provides co-stimulatory signals required for T-cell activation and survival, has been showed to raise up to 6 days prior diagnosis of AR (García-Alonso et al. 1997). An upregulation of CD28 in CD4(+) lymphocytes in the periods of greatest AR has been observed, without being influenced by hepatitis B virus (HBV), hepatitis C virus (HCV), or CMV infections, which makes CD28 useful to discriminate between AR and the cellular activation induced by viral reinfection (García-Alonso et al. 1997; Minguela et al. 2006). Later, Boleslawski et al. (2008) studying prospectively the expression of CD25, CD28, and CD38 on CD3+, CD4+, and CD8+ cells in 52 LTRs found that in addition to the increase of CD28 expressing T cells during AR, there was also a raise in the frequency of CD38 expressing T cells. However, although they did not observed an elevation of CD28+ and CD38+ T cells during infection comparing to patients with an uneventful postoperative course, they could not exclude the possibility that infections themselves might alter the expression of CD28 and CD38, because the number of patients was too small.

On the other hand, circulating CD4+CD25^{high}FoxP3+ regulatory T cells are regulatory T cells were showed to be significantly lower in liver allograft recipients with AR compared with patients without rejection. The frequency of circulating CD4+CD25highFoxP3+ T cells was also negatively correlated with the RAI ($r = -0.80$; $P < 0.01$) (He et al. 2011). These results were recently confirmed by Wang et al. (2014), who found that the frequency of circulating CD4+CD25+FoxP3+ cells decreased at the onset of AR whereas the frequency of circulating Th17 cells increased. They also observed that the Treg/Th17 ratio had a negative correlation with liver damage indices and the RAI. The increase of circulating Th17 cells during AR agrees with the study of Fan et al. (2012), as mentioned before. Thus, the Treg/Th17 ratio can be suggested as a candidate marker for the diagnosis of AR; however, there is a lack of data about the behavior of this ratio in other OLT complications.

Recently, Raschzok et al. (2015) in a prospective study with 94 LTRs observed lower CD44 and higher CXCL9 serum protein levels at day 1 posttransplantation in patients developing later AR. Even CXCL9 levels resulted higher before transplantation in these patients. CD44 values (cutoff <200.5 ng/mL) or CXCL9 values (cutoff >2.7 ng/mL) at the day after transplantation allowed to differentiate between rejection and no rejection with a sensitivity of 88% or 60% and a specificity of 61% or 79%, respectively. The combination of both biomarker cutoffs had a positive predictive value of 91% and a negative predictive value of 67% for clinically significant AR. Furthermore, CD44 levels were different in patients with graft dysfunction due to other reasons.

Toll-like receptor 4 (TLR4), with an important role in the activation of the innate immune system, has been showed to be related to acute liver rejection. Testro et al. (2011) studying 26 LTRs observed that patients experiencing AR showed higher levels of TLR4 in CD14+ cells prior to liver transplantation and a significant downregulation during the first week after transplantation.

Some cell adhesion molecules involved in the immune response have also been studied as potential biomarkers of AR. Intercellular adhesion molecule 1 (ICAM-1), cell surface glycoprotein regulating infiltration of leukocytes into the allograft during AR, E-selectin (expressed in endothelial cells recruiting leukocytes), and vascular cell adhesion protein 1 (VCAM-1) (also mediating adhesion of immune cells to the vascular endothelium) have been observed to be increased in serum of patients with AR, especially in acute steroid-resistant rejection. However, the increase of these molecules has also been observed in infectious episodes and other liver posttransplantation complications, not resulting practically as specific markers of AR (Goto et al. 1998).

Peripheral blood count of eosinophils has been suggested as a candidate biomarker for AR after liver transplantation. Foster et al. (1989) studying 60 LTRs found that blood eosinophilia (absolute eosinophil count >500 cells/mm3) occurred up to 5 days prior to AR diagnosis. In these cases of rejection, blood eosinophilia was followed by graft eosinophilia. Afterward, Barnes et al. (2003) in a cohort of 101 LTRs found that an elevated eosinophil count during or 1 day before biopsy had a positive predictive value of 82% for AR,

whereas a normal eosinophil count excluded moderate/severe rejection with a predictive value of 86%. Nevertheless, in a recent study with a larger series of patients, it was found that although peripheral eosinophil count was strongly associated with moderate/severe rejection (OR = 2.15; $P = 0.007$), the area under ROC curve was only 0.58, concluding that peripheral eosinophilia was not sufficiently predictive of moderate/severe histological rejection. However, this study showed that changes (between the first and second biopsy) in eosinophil count over time can accurately predict the histological resolution of rejection (Rodríguez-Perálvarez et al. 2012).

In a study of Yu et al. (2013), the frequency of Vδ1+ T-cell subset was reduced during AR, whereas the frequency of Vδ2+ T-cell subset increased in these patients. Therefore, a reduction of the Vδ1+/Vδ2+ ratio during AR was observed, showing a negative correlation with alanine transaminase (ALT) and alanine aminotransferase (AST) levels. On the contrary, they did not found differences in frequency of γδ T cells. Considering that in CMV and HCV and other infections, an increase of Vδ1+ T-cell and a decrease Vδ2+ T-cell subset have been observed (Puig-Pey et al. 2010), the ratio Vδ1+/Vδ2+ ratio may act as a biomarker to predict the immunological situation of recipients after liver transplantation.

On the other hand, with pediatric patients following liver transplantation, a study revealed that serum plasminogen activator inhibitor 1 (PAI-1) levels at the time of AR were significantly higher than that after the rejection ending and those on days 14 and 28 in the group without rejection (Mimuro et al. 2010). Therefore, levels of PAI -1 in pediatric patients could be useful for suspecting AR after liver transplantation. However, no more studies have been performed in this sense to corroborate these results.

Bile Immunological Markers

Apart from serum biomarkers, some studies have focused on finding possible markers of AR in bile, though results are not very conclusive.

Some of the already mentioned cytokines and cell membrane proteins in serum have also been observed in bile. In that way, bile IL-6 levels were found to be significantly increased in patients with AR, decreasing in response to antirejection therapy, but these levels were also observed elevated in patients with cholangitis (Umeshita et al. 1996).

As in serum, Lang et al. (1995) observed that biliary ICAM-1 was specifically elevated during rejection and not during infection or when no rejection was apparent; however, in a later study, this increase was also found during infectious complications (Warlé et al. 2003). In the case of the IL-2R, bile IL-2R levels are increased in LTRs with AR, although comparing to serum levels, higher specificity and selectivity is reached (Adams et al. 1989).

Apart from the inconsistent results among studies, bile biomarkers present a clear limitation to be used in the clinical practice compared to serum biomarkers; bile sampling needs invasive procedures, whereas blood or serum samples are easily acquirable and do not represent any further risk for the patient.

Future Biomarkers

Permanent advances in analytical techniques, especially in the field of -omics (genomics, transcriptomics, proteomics) during the last decades, have opened new perspectives for the discovery of biomarkers.

Genomics

More advances in the identification of genes involved in allograft rejection have been carried out in the field of kidney transplantation; however, some studies have been developed in liver transplants. In a study performed by Berberat et al. (2006), they identified six genes related to the inflammatory response that significantly correlated with the occurrence of early graft dysfunction. Higher C-reactive protein gene expression levels correlated significantly with the need of therapeutic interventions due to graft-related complications, whereas the expression of five genes related to vascular endothelial cell physiology (connective tissue growth factor, CTGF; WW domain-containing protein 2, WWP2; programmed death ligand 1, CD274; vascular endothelial growth factor, VEGF; and its receptor FLT1) was significantly reduced in biopsies of patients with graft-related complications in the first month. Authors, using a risk score based on the expression of these five genes, determined that early allograft dysfunction could be predicted with 96% sensitivity. Nevertheless, authors did not differ among causes of allograft dysfunction, so these genes cannot be associated directly to AR.

Apart from wide genome studies, the polymorphisms of some genes related to the immune response have been identified to be associated with AR after OLT. In that way, Yu et al. (2014) in a study with 289 LTRs analyzing preoperative peripheral blood DNA of recipients found that interferon regulatory factor 5 (IRF5), which transcriptionally activates inflammatory cytokines, was genetically related to AR and could be considered a risk factor for AR after liver transplantations. In brief, the IRF5 gene polymorphism rs3757385 was found to be associated with AR, and homozygous individuals for this polymorphism were at higher risk of AR. However, polymorphism was not studied in patients with other complications after OLT.

Oetting et al. (2012), analyzing 37 different single nucleotide polymorphisms within the toll-like receptor 4 (TLR4) gene in 738 recipients LTRs, found that various donor polymorphisms of the TLR4 gene were clearly associated with AR and could be considered risk factors of graft loss. That would be in agreement with results of Testro et al. (2011), as mentioned before, who observed that levels of expression TLR4 in CD4+ (at protein level) cells were related to AR.

Transcriptomics

Asaoka et al. (2009), by microarray analysis of liver biopsies RNA, demonstrated novel transcriptome patterns for AR with recurrent hepatitis C and different from those in recipients with only recurrent hepatitis C without AR, suggesting that gene expression profiling may be useful in the diagnosis of AR in recipients with hepatitis

C. In their study, they found 126 relatively overexpressed genes in the ACR, 15 of them involved in the inflammatory and immune response and antigen presentation.

Meanwhile, in peripheral blood, higher gene expression levels of guanylate-binding protein 2 (GBP2) and interferon regulatory factor 1 (IRF1) (genes mainly expressed in leukocytes) were observed in samples of patients with AR comparing with those from patients with other liver dysfunctions and normal liver after transplantation, but only GBP2 expression resulted significant. Using a cutoff of 20, the sensitivity and specificity of GBP2/GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were 63% and 85%, respectively; as a result, authors concluded that GBP2 may be useful for the diagnosis of AR in patients with liver dysfunction after liver transplantation (Kobayashi et al. 2010).

On the other hand, circulating miRNAs that are extremely stable and protected from RNAses have emerged as candidate biomarkers for any disease. They fulfill many characteristics of ideal biomarkers: noninvasive, stable, and easily detected. In this sense, different studies have showed that some circulating miRNAs are associated with AR episodes after liver transplantation (Farid et al. 2012; Wei et al. 2013), some of them related to the immune system. As lymphocyte infiltration into the graft is a major pathological feature of AR after liver transplantation, the contribution of lymphocyte miRNAs during graft rejection have been evaluated. Wei et al. (2013) found that regulation of miR-142-3p expression in lymphocytes may impact graft outcomes.

In a previous study, serum levels of hepatocyte-derived miRNAs, miR-122, miR-148, and miR-194, correlated with hepatic injury and AR (Farid et al. 2012). In fact, expression was elevated earlier than aminotransferase levels during AR.

Nevertheless, miRNA have also been associated with other infectious diseases, as HBV and HCV, among them miRNA-122. Thus, although miRNA biomarkers clearly have potential for clinical application in the setting of liver transplantation, the number of studies on this topic should be expanded and validations should be carried out.

Proteomics

Considering that a wide range of proteins are related to the immune response in AR, proteomics seems a promising approach to determine new biomarkers.

Recently, Massoud et al. (2011) carried out a serum proteome profile in histologically confirmed AR patients, and afterward those identified proteins were validated by ELISA in another cohort of patients. This study showed that complement component 4 (C4) and complement component 1q (C1q) were both independent predictors of AR. C4 had the greatest predictivity for differentiating patients with or without AR (sensitivity = 97%; specificity = 62%; positive predictive value = 74%; negative predictive value = 94%). Combining levels of C4 and ALT improved these results (to 96%, 81%, 86% and 94%, respectively). So, serum C4 and ALT levels could be highly predictive of AR in LTRs; however, because of the reduced number of patients, this study should be validated in a larger multicenter trial.

Currently, only two noninvasive tests, approved by the US Food and Drug Administration, are commercialized. AlloMap (CareDx, San Francisco, USA) is

intended to assess AR after heart transplantation, determining gene expression profile of RNA isolated from peripheral blood mononuclear cells (PBMC). ImmuKnow (Viracor-IBT Laboratories, Lee's Summit, MO, USA) detects cell-mediated immunity in immunosuppressed patients. The assay detects cell-mediated immunity by measuring the concentration of ATP from CD4 cells following stimulation. A systematic review and meta-analysis carried out by Rodrigo et al. (2012) concluded that the ImmuKnow test was a valid tool for determining the risk of further infection in adult LTRs, but the elevated heterogeneity across studies did not allow to conclude the usefulness of ImmuKnow to predict AR. In a recent study of Ravaioli et al. (2015), results showed that ImmuKnow can provide additional data which can help to optimize immunosuppression and improve patient outcomes after liver transplantation, rather than discriminate the risk of AR.

Biomarkers of Chronic Rejection

Chronic liver rejection can be defined as an immunologic injury to the graft, which occurs after severe or persistent AR and results in irreversible loss of bile ducts, arteries, and veins (Neumann et al. 2002). Histologically, CR is characterized by destruction of interlobular bile ducts associated with cholestasis. Normally, it is associated with centrilobular inflammation and necrosis and with foam cell lesion within intrahepatic arterial branches. CR rejection can progress to bridging fibrosis and cirrhosis (Neuberger 1997).

There has been a progressive decrease in the prevalence of CR, being around 5–15%, and nowadays accounts for less than 2% of cases of graft failure. CR leading to re-transplantation is relatively rare, around 5%. However CR is probably underestimated due to the absence of routinely performed liver biopsies on the long term in most centers (Adams et al. 2015). The onset of the disease is during the first year posttransplant and occurs only in rare cases in the long term after OLT (Neuberger 1997). Late CR might also develop after a therapy refractory late acute rejection episode, normally in therapy noncompliant patients.

Because CR is a potentially reversible pathologic state at its onset, early diagnosis is very important. Generally, histopathologic diagnosis is carried out when CR is well established and results are irreversible; so as in AR, noninvasive diagnostic tools for early diagnosis of CR would be very valuable for the treatment of CR. Comparing with AR, few studies have been performed in the case of CR in order to study possible biomarkers, and all of them have been carried on liver biopsies, with no studies about biomarkers in serum. Thus, independent of the results, noninvasive procedures are not provided for CR diagnosis.

C4d has been proposed as a marker of CR. In a study of Lorho et al. (2006), searching for the presence of C4d in posttransplant hepatic biopsies, found that C4d expression appeared in 100% of biopsies classified as CR and in 33% of biopsies diagnosed as AR, being absent in biopsies of patients with recurrent hepatitis C infection without rejection.

The increase of p21 WAF1/Cip1 in liver biopsies has also been associated with early CR and suggested as a marker of this complication (Lunz et al. 2001). The percentage of p21 WAF1/Cip1 biliary epithelial cells (BECs) and the number of p21 WAF1/Cip1BECs per portal tract are significantly increased in early CR compared to BECs in normal liver allograft biopsies or those with nonspecific changes, chronic hepatitis C, or obstructive cholangiopathy. In fact, successful treatment of early CR is associated with a decrease in the percentage of p21 WAF1/Cip1BECs and the number of p21 WAF1/Cip1BECs per portal tract.

Recently, Wei et al. (2015) carried out a study in a rat model of CR after liver transplantation. In order to explore possible biomarkers of the disease, they performed a proteomic analysis of grafts samples 120 days after operation. Authors found that expression of 62 proteins significantly changed in CR. From them, finally they identified clusterin (CLU), lipocalin 2 (LCN2), and cytokeratin 19 (Krt19) as early and reliable biomarkers for chronic rejection on liver biopsies. Expressions of CLU and LCN2, a neutrophil gelatinase-associated lipocalin, were found to be upregulated both in AR and CR but more upregulated in CR. Krt19 expression was downregulated probably because of the disappearance of interlobular bile ducts.

DSAs: Potential Biomarkers for Allograft Rejection?

The adverse impact of donor-specific human alloantibodies (DSAs) in solid organ allografts has been widely demonstrated, such as the kidney, pancreas, or heart (Kaneku et al. 2013). Until recently, this impact has been considered irrelevant and ignored in OLT outcomes, since liver allografts possess some degree of alloantibody resistance (Pons et al. 2011). However, in the last few years, detailed studies have shown alloantibody-mediated adverse consequences in liver allografts (Castillo-Rama et al. 2008; Kozlowski et al. 2011; Kaneku et al. 2013), questioning the impact of DSAs on short- and long-term liver transplant outcomes. Currently, it is assumed that antibody-mediated rejection (AMR) can occur in the liver allograft, being involved in both acute and chronic rejections (Adams et al. 2015). Results from recent studies suggest that the presence of preformed or de novo DSAs is associated to AMR and a decrease in the liver allograft survival (Kozlowski et al. 2011; Kaneku et al. 2013). Nevertheless, not all DSAs have been seen to produce the same response after OLT and the mere presence of DSAs has entailed allograft injury, so that DSA concentration and DSA type seem to play an important role in the development of allograft injury and liver transplant outcomes. Considering these findings, certain circulating anti-HLA antibodies could be also suggested as potential biomarkers for the managing of immunosuppression after OLT.

DSAs and Liver Allograft Rejection

Only when AMR is accurately diagnosed early and successfully treated can graft outcomes improve because a delay in the diagnosis of AMR usually results in substantial allograft injury or failure. To date, liver acute AMR is diagnosed based on the following criteria: (1) the presence of DSAs in serum, (2) histopathologic

evidence of diffuse microvascular endothelial cell injury and microvasculitis, (3) strong and diffuse C4d detection in tissue, and (4) reasonable exclusion of other causes of injury that might result in similar clinical signs. On the contrary, criteria for diagnosis of chronic AMR need to be further studied, although subsinusoidal and perivenular fibroses, associated with DSA and C4d staining, have been already described (O'Leary et al. 2014a). So, before liver biopsy, determination of serum DSAs can allow the identification of patients in higher risk of AMR after liver transplantation; however, literature data pointed some considerations in order to use them as biomarkers of liver allograft rejection.

Although some studies have shown that the presence of preformed DSAs was not always associated to a decrease in the liver allograft survival rate or to a worse liver transplant outcome, recently, others have shown alloantibody-mediated adverse consequences in liver allografts (Castillo-Rama et al. 2008; Kozlowski et al. 2011; Kaneku et al. 2013). Thus, a clear controversy exists in the elucidation of the role of DSAs on the liver allograft injury and rejection.

Differences in preformed DSAs levels have been suggested as one of the reasons for the differences of effects between patients and the consequent appearance of clinically significant liver allograft injury and possible AMR. O'Leary et al. (2014b) observed LTRs with high preformed DSAs with higher mean fluorescence intensity (MFI) in its detection seemed to be at higher risk of suffering substantial early graft injury and consequent AMR.

Scornik et al. (2001) quantified by flow cytometry preformed IgG antibodies against donor cells in 465 LTRs and found that the incidence of rejection did not significantly differ between antibody-positive and antibody-negative patients. However, patients with higher antibody concentrations showed higher percentage of steroid-resistant rejection (31% at 1 year) than patients with lower antibody concentration (4%) or without antibodies (8%). These effects were mainly due to T-cell (HLA class I) antibodies. These authors concluded that the effect of preformed DSA on liver allograft outcomes depended on antibody concentration and the patient response to steroid treatment.

In another retrospective single-center study comprising 896 liver transplants, preformed HLA class I and II antibodies, detected by both complement-dependent cytotoxicity and multiple bead assay (Luminex xMAP), were found to be associated with shorter graft survival within the first year posttransplant, but in patients with anti-HLA class I antibodies, the decreased survival rate disappeared after the first year posttransplant and patients with preformed anti-HLA class II antibodies showed lower graft survival 5-year posttransplant (Castillo-Rama et al. 2008). These results are in accordance with those from the study of O'Leary et al. (2013) who observed that preformed class II DSAs were associated with an increased risk of early rejection.

Moreover, among DSAs, IgG subclasses have also been shown to have different effects on liver allograft outcomes. DSAs in chronic rejection patients have been found to be more often of multiple IgG subclasses including IgG3 compared to control group where most DSAs were of a single IgG subclass and without IgG3 (Kaneku et al. 2013). Recently, a retrospective study evaluating 1270 LTRs

demonstrated the inferior survival associated with IgG3-positive DSA-positive patients and C1q-positive DSA-positive patients compared to DSA-negative patients. IgG3-positive DSA-positive patients had the highest hazard ratio for death, but its only analysis could not exclude standard DSA test since IgG3-negative DSA-positive patients remained at increased risk of death compared to DSA-negative patients. Nonetheless, IgG3 analysis is suggested as an independent predictor of allograft outcome. Meanwhile, the same study suggested that C1q-fixing DSA test could identify patients with preformed high MFI DSAs, who have been shown to have higher risk of rejection (O'Leary et al. 2015).

Apart from that, most studies have focused on preformed HLA antibodies, and little attention has been paid to de novo DSAs. Kaneku et al. (2013) considering 749 LTRs found that 8.1% of patients developed de novo DSAs 1 year after transplant, almost all de novo DSAs were against HLA class II antigens, and these patients had significantly lower patient and graft survival. This negative relation of de novo DSA and liver graft have been shown by others (Kozlowski et al. 2011).

Therefore, not all DSAs and levels produce the same effects on liver allograft, so differentiation among them should be made in order to be possible candidates as biomarkers of AMR. To date, literature shows that high preformed DSA levels, HLA class II DSAs, IgG3, and de novo DSAs are associated to allograft injury and AMR and could be suggested as biomarkers of AMR in LTRs. However, the development of analytical techniques for a further and more accurate discrimination and quantification of DSAs is required in order to progress in this field.

Current DSA Analytical Techniques

Techniques to detect DSAs have advanced spectacularly because of renal allograft transplantation. Firstly, cytotoxic crossmatch assay was used; however limited sensitivity and specificity did not allow to distinguish HLA from non-HLA antibodies, although performed pretransplantation allows to eliminate hyperacute renal allograft rejection.

This initial test was then supplemented by flow cytometry and solid-phase immunoassays (SPIs), such as ELISA, ELISPOT, and LUMINEX®. Flow cytometry lets differentiate HLA and non-HLA DSAs, even though it shows low sensitivity and specificity to characterize all HLA alloantibodies. This has been solved with the SPI technology based on the multi-analyte bead assays performed on LUMINEX® platforms. However, the latter assays can be influenced by substances in serum and reproducibility among lots and centers could be a considerable constraint. For these reasons, today cell-based assays and SPI are frequently performed in parallel (O'Leary et al. 2014a). In fact, Leonard et al. (2013) outlined an HLA antibody test algorithm for LTRs. Briefly, pretransplant patients were suggested for the analysis of HLA antibodies' presence by solid-phase testing. If HLA antibodies' presence resulted positive, a crossmatch by flow cytometry at the time of transplantation was recommended. Then, if a strongly positive crossmatch was detected (median channel shift, MCS $>$ 200), evaluation of DSAs levels with both flow cytometry

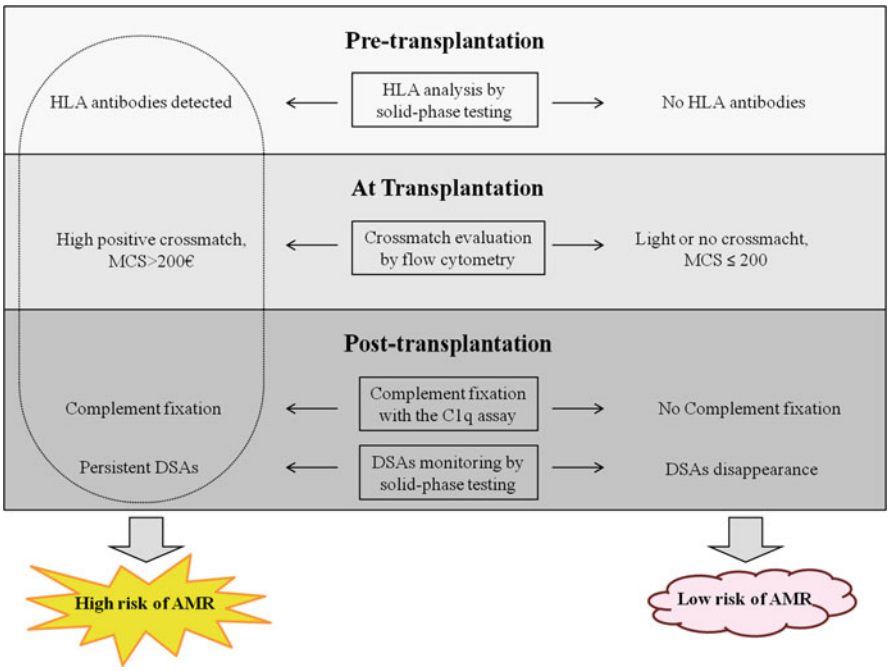

Fig. 2 Risk of AMR in LTRs. Monitoring antibody-mediated rejection in liver transplant recipients according to Leonard et al. (2013). Those authors proposed sequential analyses (pre- and posttransplantation) in liver transplant recipients in order to evaluate the risk of suffering antibody-mediated rejection; *AMR* antibody-mediated rejection, *DSAs* donor-specific human antibodies, *HLA* human leukocyte antigen, *MCS* median channel shift, *C1q* complement component 1q

(MCS) and Luminex (MFI) was further accomplished. Thereafter, resulting the crossmatch strongly positive because of high levels of DSAs, DSAs should be tested for complement fixation and monitored after transplantation. These authors concluded that a strongly positive crossmatch, positive complement fixation, and persistent posttransplantation DSAs indicated an increased risk for AMR (Fig. 2).

Recently, molecular phenotyping has also been proposed as a technique to be able to differentiate patients with DSAs in serum who do not experience pathologic injury from those with DSA, experiencing subclinical pathologic injury that becomes apparent later, and those with clinically evident pathologic injury, opening a promising field in the use of DSAs in the monitoring of liver transplantation (O'Leary et al. 2014a).

There is an increasing need to characterize DSAs more accurately in order to clarify their role in AMR in liver transplantation and for the early diagnosis of AMR. That entails the development of new cross-platform analyses that can include routine and multiplex protein immunohistochemistry, messenger RNA and miRNA expression arrays, and proteomics and metabolomics techniques.

Immunological Biomarkers of Graft Acceptance/Tolerance

Allograft Tolerance

Successful immunosuppression (IS) withdrawal in LTRs occurs more frequently than in other solid organ transplantations. It has been showed both in noncompliant patients and in those with serious complications related to immunosuppression (IS) drugs, like lymphoproliferative disease (Lerut and Sánchez-Fueyo 2006). Thus, the liver is considered as an immunologically privileged organ by its intrinsic tolerogenic properties that make it the most amenable graft to IS withdrawal.

Literature data provides a percentage of successful weaning at around 20–33%, although the prevalence could be higher in pediatric population under 1 year, where this percentage could reach 64% (Li et al. 2012), and in adult recipients with more than 10 years of posttransplant follow-up (Sánchez-Fueyo 2011). This phenomenon is known as spontaneous operational tolerance, and these patients are considered as "operationally" tolerant recipients.

In the clinical setting, the state of tolerance can be achieved in two different ways: spontaneously or induced. The endpoint can be the complete IS drug withdrawal (operational tolerance) or minimization of this ("prope" tolerance), which is the most frequent situation in clinical practice (Table 2). To date, it is unknown whether minimally immunosuppressed patients actually will be able to complete the discontinuation of IS without developing rejection (Lerut and Sánchez-Fueyo 2006). Neither is clear whether "prope" tolerance is a previous step of operational tolerance or two independent conditions.

Since IS withdrawal has an inherent risk of AR, identifying biomarkers of graft acceptance is required in order to tailor IS therapy after liver transplantation (Germani et al. 2015).

Table 2 Tolerance definition

Immunological tolerance	Absence of an alloimmune response toward a specific antigen without immunosuppression therapy
Operational tolerance	Absence of acute or chronic rejection with normal function and histology in immunocompetent recipients who discontinue conventional immunosuppression for more than a year
Prope tolerance (almost tolerance)	Graft acceptance using very low doses of immunosuppressive drugs
Central tolerance	Mechanism based on the theory of donor antigens recognized as antigens of "self". (performed transplanting donor hematopoietic cells to induce intrathymic clonal deletion of T precursor cells expressing T cell receptor)
Peripheral tolerance	Induction of tolerance by pharmacological immunosuppression of self-reactive T cells in periphery (Girmanova et al. 2015)

Types of allograft tolerance depending on the immunological response and the immunosuppression regimen after liver transplantation

Table 3 Findings in tolerant LTRs

Sample	Technique		Potential biomarkers
	Flow cytometry	Microarray/RT-PCR	
Peripheral blood	Increase in CD4+ $CD25^{high}$ cells	Upregulation of FoxP3 expression	Regulatory T cells
	Increase in CD4+ $CD25^{high}$ $CD127^{low}$ cells		
	Increase in CD4+CD25++ cells		
	Increase in γδ1/γδ2 T cell ratio	Upregulation of γδTCR+ T cell related genes expression	γδTCR+ cells
	Increase in pDC/mDC ratio		Dendritic cells
	Increase in HLA-G expression on mDC		
		Upregulation of NK related gene expression	NK cells
Liver tissue		Upregulation of FoxP3 expression intragraft	Regulatory T cells

Findings in peripheral blood or liver tissue of tolerant liver transplant recipients (LTRs) are shown. Potential biomarkers of graft tolerance have been proposed according to those findings and different analytical techniques (flow cytometry and Microarray/RT-PCR) have been used

Biomarkers of Graft Acceptance

In order to identify biomarkers of graft acceptance, several studies have employed blood samples of operationally tolerant recipients to perform gene expression profiling and immunophenotyping. In recent years, attention has been directed toward samples of liver tissue to find immune parameters associated with operational tolerance. The interest generated has made possible to identify several potential markers of allograft tolerance (Table 3).

NK Cells

Studies using gene expression analysis demonstrate that NK cells and related transcripts are upregulated in blood samples from operationally tolerant LTRs, determined by microarray and real-time PCR platforms. In fact, some authors, like Londoño et al. (2012), assert that NK-related transcripts seem to be the most robust markers of operational tolerance. These results have been confirmed not only in adults but also in pediatric LTRs (Li et al. 2012) and in operationally tolerant kidney recipients (Martínez-Llordella et al. 2007). Indeed, according to Bohne et al. (2012), tolerant recipients exhibit an expansion of NK cells in peripheral blood even before the initiation of drug minimization.

γδTCR+ Cells

Similar to NK cell-related transcripts, genes encoding for gamma-delta T cells (γδT-cell) and for proteins involved in the cycle cell proliferation arrest are upregulated in tolerant liver recipients compared to immunosuppression-dependent patients or

healthy individuals. So they appear to be specifically related to the tolerant state and their expression seems to be independent on either HCV infection or IS treatment (Martinez-Llordella et al. 2008).

There are two kinds of $\gamma\delta TCR^+$ T-cell subsets in human peripheral blood; $\gamma\delta 2TCR^+$ T cells account for more than 70–80% of circulating T cells in healthy individuals, while $V\delta 1TCR^+$ subtype preferentially populates epithelial tissues such as the intestine, liver, and spleen (Martinez-Llordella et al. 2008). In contrast, a significant increase γδ1/γδ2 T-cell ratio has been found in operationally tolerant liver-transplanted patients when compared with liver-transplanted patients on immunosuppression and with age-matched healthy controls (Martinez-Llordella et al. 2007). Along these lines, Bohne et al. (2012) show a decreased proportion of γδ 2-TCR cells in tolerant patients as compared with non-tolerant recipients before the start of IS withdrawal.

Regulatory T Cells

Regulatory T cells (Treg cells) are characterized by the coexpression of CD4, CD25 (interleukin 2 [IL2] receptor α chain), and Forkhead box 3 (FOxP3) (Pons et al. 2008).

Several studies have shown an expansion of $CD4^+CD25^{high}$ T cells in phenotypic analysis of PBMC of tolerant recipients for more than 2 years after liver transplantation than either non-tolerant patients or healthy individuals, but this increase does not seem to be present at the beginning of weaning. Castellaneta et al. (2011) suggest that $CD4^+CD25^{high}CD127^{low}$ would provide a better marker for tolerance.

Pons et al. (2008) described an increase of FoxP3 mRNA in blood samples of tolerant recipients and observed that FoxP3 intragraft transcript levels were 3.5-fold increase up to the beginning of the tolerance phenomenon, which is continued at the end of therapy. Li et al. (2008), following the same line, reported that FoxP3 mRNA is also higher in liver biopsies from tolerant transplanted patients compared with patients on immunosuppression, but mRNA levels were similar in operationally tolerant and chronic rejectors. However, while some authors report increased FoxP3+ transcript levels in peripheral blood and liver tissue of tolerant recipients, other authors did not observe differences between tolerant and non-tolerant patients, neither Foxp3 transcripts nor immunophenotyping analysis (Bohne et al. 2012).

It is noteworthy that Treg cell suppressor function and their survival depend on the presence of IL-2. Calcineurin inhibitors block IL-2 production, which may thereby negatively affect the homeostasis of Treg cells. Several reports support this hypothesis, as Pons et al. (2008) indicate. Therefore, up until today, the role of Treg cells in graft acceptance is less clear because the use of immunosuppressive drugs could alter their expression and by the disparity in the results found.

Dendritic Cells and HLA-G Expression

Dendritic cells (DC) are innate immune system cells that are also important in the regulation of adaptive immunity, including the ability to induce regulatory T cells (Treg cells). There are two types of DC: monocytoid DC ($CD11c^+$), which induce Th1 cell differentiation in vitro, and plasmacytoid DC ($CD123^+$), which promote

Th2 cell responses. They are designated as DC1 (mDC) and DC2 (pDC), respectively.

Mazariegos et al. (2005) described an increased pDC/mDC ratio in tolerant recipients. According to their results, Reding et al. (2006) speculated that a pDC1/pDC2 subset ratio of 0.1 could serve as the threshold above which a patient might be considered for IS weaning. However, differences in the distribution of pDC in operationally tolerant LTRs have not been confirmed by all studies (Martinez-Llordella et al. 2007).

Moreover, mDC express higher levels of histocompatibility antigen, class I, G (HLA-G) in operational recipients than in IS-dependent patients or healthy controls, and these data are independent on the kind and dose of immunosuppressive drug. In addition, increased Foxp3 expression in Treg LTRs tolerant operationally correlated with the level of HLA-G expressed by mDC (Mazariegos et al. 2005).

Potential Applications to Prognosis, Other Diseases, or Conditions

AR and CR represent important complications in the managing and prognosis of OLT. After clinical suspicion, their diagnosis supposes the histopathological evaluation of liver biopsies. Liver biopsies are costly, result in being insidious, and represent a risk for patients; thus, the finding of biomarkers allowing the early detection of these liver transplantation complications, and their differentiation from others with similar clinical signs and biochemical alterations, will be of important value for the monitoring and the better immunosuppression managing of LTRs and therefore in the prognosis of the OLT outcome.

The review of the different potential immunological biomarkers that have been evaluated in the literature shows that most of the obtained results are inconclusive, either because biomarkers have not been studied in patients with other OLT complications, or, despite of doing it, they have showed similar patterns hampering the differentiation of rejection from other OLT complications; have been achieved from a reduced number of patients, since more studies, including higher number of patients, are needed; and require further validation, with the performance of prospective independent larger multicenter trials. Few of the reviewed biomarkers fulfill all these criteria and can be honestly considered as future potential AR biomarkers to be set up in the clinical practice. The most valuable of the reviewed biomarkers are summarized in Table 4. Despite the promising results of these biomarkers, its application to the clinics should entail further studies and validations.

Contrary to AR, where significant advances have been carried out in the discovery of biomarkers, in CR there is a considerable lack of studies in this sense, maybe because of its low prevalence and the variability among patients, which makes it impossible to suggest any future potential biomarker to diagnoses this complication. Therefore, the discovery of future noninvasive biomarkers of CR is a worthy field to investigate.

Table 4 Valuable immunological biomarkers for AR

	Reference	Discrimination from other liver dysfunctions	Prospective; sample size
CD44	Raschzok et al. (2015)	Yes	Yes; 94 LTRs
Treg/Th17 cells ratio	Wang et al. (2014)	No	Yes; 38 LTRs
CD28+ T cells	Minguela et al. (2006)	Yes	Yes; 237 LTRs
CD28+C38+ T cells	Boleslawski et al. (2008)	Yes	Yes; 52 LTRs
Eosinophilia	Foster et al. (1989)	Yes	Yes; 60 LTRs
	Barnes et al. (2003)	Yes	Yes; 101 LTRs
IL-17	Fábrega et al. (2009)	No	Yes; 50 LTRs
	Fan et al. (2012)	No	Yes; 76 LTRs

Most promising potential biomarkers of acute rejection (AR) that have been identified in liver transplant recipients (LTRs) are presented

On the other hand, advances and studies using new techniques, in the fields of genomics, transcriptomics, and proteomics, have provided new potential biomarkers. And despite the few studies developed and the number of patients included, promising results have been obtained, especially in the field of proteomics. For sure, the revolution of the -omics era will allow the finding of more robust, sensitive, and specific biomarkers.

In relation to AMR of liver allografts, the identification of the impact of preformed and de novo DSAs in AMR would allow to differentiate this kind of complication that until now had been mistaken with other OLT complications, as AR. Moreover, preformed DSA analyses would help to predict LTRs at risk of rejection before transplant, percentage of graft survival, and therefore OLT outcome. As well, after transplantation, certain DSA identification would allow to identify patients that need a closer monitoring, in order to prevent rejection, before irreversible allograft injury is developed. However, the controversy of some studies makes mandatory the performance of further prospective multicenter studies to identify the class of DSAs implicated in AMR. Moreover, advances in analytical techniques will permit to progress in the use of DSAs. In the next years, DSAs will be very useful in the prognosis of liver transplantation outcomes, even prior to transplant. In other solid organ transplantations, as the kidney or heart, DSAs are widely accepted as a risk factor for decreased graft survival, and their determination helps to predict transplantation outcomes.

With regard to allograft tolerance, establishing biomarkers related with graft acceptance would allow assessing the real suppression state of the immune system after liver transplantation. In this way, transplant clinicians could modulate the immunosuppressive therapy depending on patients' needs and identify liver

transplant recipients who can discontinue or reduce the dosage of IS drugs without graft rejection. Indeed, this could allow the choice of immunosuppressor molecule, dosage adjustment, and target therapeutic window. Furthermore, establishing biomarkers of operational tolerance may provide tools to determine endpoints for tolerance induction trials, provide biological basis for guiding IS weaning protocols, and predict the success of withdrawal.

Against this background, the routine implementation of biomarkers and personalized therapy in patients would enable to revolutionize the quality of life of transplant recipients, with less exposure to toxicity or other adverse effects associated with immunosuppressive treatment, as well as a reduction in drug costs.

Summary Points

- Biomarkers of liver allograft rejection and tolerance would be very useful in the managing of LTRs after transplantation, which would considerably improve liver transplantation outcomes.
- Multiple potential biomarkers for liver AR, related to the immune system, have been studied; however, few of them permit to distinguish AR from other OLT complications or have been validated in later prospective multicenter trials.
- There is a lack of studies about potential biomarkers for liver allograft CR, maybe because of its low prevalence in LTRs; however, considering that CR can be underestimated, it will be very worthy to invest efforts in their study.
- As the role of DSAs (types of DSAs, levels needed, analyses) in liver AMR is elucidated, their use as biomarkers will allow to recognize LTRs in risk of suffering AMR, even before transplant, which will improve allograft survival and OLT outcomes.
- Some potential biomarkers of allograft tolerance, as NK cells, have been identified. They will allow a better managing of patient immunosuppression reducing adverse effects of immunosuppressors.
- Data available for biomarkers of graft acceptance are more encouraging compared to biomarkers of AR.
- Development of analytical techniques will allow finding more specific and sensitive biomarkers for liver allograft rejection and tolerance.
- More prospective and multicenter trials are required before the reliable implementation of biomarkers of rejection and tolerance in the clinical practice of LTRs.

References

Abraham SC, Furth EE. Receiver operating characteristic analysis of serum chemical parameters as tests of liver transplant rejection and correlation with histology. Transplantation. 1995;59:740–6.

Adam R, Karam V, Delvart V, et al. Evolution of indications and results of liver transplantation in Europe. A report from the European Liver Transplant Registry (ELTR). J Hepatol. 2012;57:675–88. doi:10.1016/j.jhep.2012.04.015.

Adams DH, Wang L, Hubscher SG, Elias E, Neuberger JM. Soluble interleukin-2 receptors in serum and bile of liver transplant recipients. Lancet. 1989;1:69–71.

Adams DH, Sanchez-Fueyo A, Samuel D. From immunosuppression to tolerance. J Hepatol. 2015;62:S170–85. doi:10.1016/j.jhep.2015.02.042.

Afzali B, Lombardi G, Lechler RI. Pathways of major histocompatibility complex allorecognition. Curr Opin Organ Transplant. 2008;13:438–44. doi:10.1097/MOT.0b013e328309ee31.

Akoglu B, Kriener S, Martens S, et al. Interleukin-2 in CD8+ T cells correlates with Banff score during organ rejection in liver transplant recipients. Clin Exp Med. 2009;9:259–62. doi:10.1007/s10238-009-0042-4.

Asaoka T, Kato T, Marubashi S, et al. Differential transcriptome patterns for acute cellular rejection in recipients with recurrent hepatitis C after liver transplantation. Liver Transpl. 2009;15:1738–49. doi:10.1002/lt.21883.

Barnes EJ, Abdel-Rehim MM, Goulis Y, et al. Applications and limitations of blood eosinophilia for the diagnosis of acute cellular rejection in liver transplantation. Am J Transplant. 2003;3:432–8.

Berberat PO, Friess H, Schmied B, et al. Differentially expressed genes in postperfusion biopsies predict early graft dysfunction after liver transplantation. Transplantation. 2006;82:699–704.

Bohne F, Martínez-Llordella M, Lozano JJ, et al. Intra-graft expression of genes involved in iron homeostasis predicts the development of operational tolerance in human liver transplantation. J Clin Invest. 2012;122:368–82. doi:10.1172/JCI59411.

Boleslawski E, Conti F, Sanquer S, et al. Defective inhibition of peripheral CD8+ T cell IL-2 production by anti-calcineurin drugs during acute liver allograft rejection. Transplantation. 2004;77:1815–20.

Boleslawski E, BenOthman S, Grabar S, et al. CD25, CD28 and CD38 expression in peripheral blood lymphocytes as a tool to predict acute rejection after liver transplantation. Clin Transpl. 2008;22:494–501. doi:10.1111/j.1399-0012.2008.00815.x.

Castellaneta A, Mazariegos GV, Nayyar N, Zeevi A, Thomson AW. HLA-G level on monocytoid dendritic cells correlates with regulatory T cell Foxp3 expression in liver transplant tolerance. Transplantation. 2011;91:1132–40. doi:10.1097/TP.0b013e31821414c9.

Castillo-Rama M, Castro MJ, Bernardo I, et al. Preformed antibodies detected by cytotoxic assay or multibead array decrease liver allograft survival: role of human leukocyte antigen compatibility. Liver Transpl. 2008;14:554–62. doi:10.1002/lt.21408.

Conti F, Calmus Y, Rouer E, et al. Increased expression of interleukin-4 during liver allograft rejection. J Hepatol. 1999;30:935–43.

Conti F, Frappier J, Dharancy S, et al. Interleukin-15 production during liver allograft rejection in humans. Transplantation. 2003;76:210–6.

Dienstag JL, Cosimi AB. Liver transplantation – a vision realized. N Engl J Med. 2012;367:1483–5. doi:10.1056/NEJMp1210159.

Fábrega E, López-Hoyos M, San Segundo D, Casafont F, Pons-Romero F. Changes in the serum levels of interleukin-17/interleukin-23 during acute rejection in liver transplantation. Liver Transpl. 2009;15:629–33. doi:10.1002/lt.21724.

Fan H, Li LX, Han DD, Kou JT, Li P, He Q. Increase of peripheral Th17 lymphocytes during acute cellular rejection in liver transplant recipients. Hepatobiliary Pancreat Dis Int. 2012;11:606–11.

Farid WR, Pan Q, van der Meer AJ, et al. Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. Liver Transpl. 2012;18:290–7. doi:10.1002/lt.22438.

Foster PF, Sankary HN, Hart M, Ashmann M, Williams JW. Blood and graft eosinophilia as predictors of rejection in human liver transplantation. Transplantation. 1989;47:72–4.

García-Alonso AM, Minguela A, Muro M, et al. CD28 expression on peripheral blood T lymphocytes after orthotopic liver transplant: upregulation in acute rejection. Hum Immunol. 1997;53:64–72.

Germani G, Rodriguez-Castro K, Russo FP, et al. Markers of acute rejection and graft acceptance in liver transplantation. World J Gastroenterol. 2015;21:1061–8. doi:10.3748/wjg.v21.i4.1061.

Girmanova E, Hruba P, Viklicky O. Circulating biomarkers of tolerance. Transplant Rev. 2015;29:68–72. doi:10.1016/j.trre.2015.01.003.

Goto S, Noguchi T, Lynch SV, et al. Is regular measurement of adhesion molecules and cytokines useful to predict post-liver transplant complications? Transplant Proc. 1998;30:2975–6.

He Q, Fan H, Li JQ, et al. Decreased circulating CD4+CD25highFoxp3+ T cells during acute rejection in liver transplant patients. Transplant Proc. 2011;43:1696–700. doi:10.1016/j.transproceed.2011.03.084.

Kaneku H, O'Leary JG, Banuelos N, et al. De Novo donor-specific HLA antibodies decrease patient and graft survival in liver transplant recipients. Am J Transplant. 2013;13:1541–8. doi:10.1111/ajt.12212.

Kita Y, Iwaki Y, Demetris AJ, Starzl TE. Evaluation of sequential serum interleukin-6 levels in liver allograft recipients. Transplantation. 1994;57:1037–41.

Kobayashi S, Nagano H, Marubashi S, et al. Guanylate-binding protein 2 mRNA in peripheral blood leukocytes of liver transplant recipients as a marker for acute cellular rejection. Transpl Int. 2010;23:390–6. doi:10.1111/j.1432-2277.2009.00991.x.

Kozlowski T, Rubinas T, Nickeleit V, et al. Liver allograft antibody-mediated rejection with demonstration of sinusoidal C4d staining and circulating donor-specific antibodies. Liver Transpl. 2011;17:357–68. doi:10.1002/lt.22233.

Lalli E, Meliconi R, Conte R, et al. Serum markers of immune activation and liver allograft rejection. Dig Dis Sci. 1992;37:1116–20.

Lang T, Krams SM, Villanueva JC, Cox K, So S, Martinez OM. Differential patterns of circulating intercellular adhesion molecule-1 (cICAM-1) and vascular cell adhesion molecule-1 (cVCAM-1) during liver allograft rejection. Transplantation. 1995;59:584–9.

Leonard GR, Shike H, Uemura T, et al. Liver transplantation with a strongly positive crossmatch: case study and literature review. Liver Transpl. 2013;19:1001–10. doi:10.1002/lt.23694.

Lerut J, Sanchez-Fueyo A. An appraisal of tolerance in liver transplantation. Am J Transplant. 2006;6:1774–80.

Li Y, Zhao X, Cheng D, et al. The presence of Foxp3 expressing T cells within grafts of tolerant human liver transplant recipients. Transplantation. 2008;86:1837–43. doi:10.1097/TP.0b013e31818febc4.

Li L, Wozniak LJ, Rodder S, et al. A common peripheral blood gene set for diagnosis of operational tolerance in pediatric and adult liver transplantation. Am J Transplant. 2012;12:1218–28. doi:10.1111/j.1600-6143.2011.03928.x.

Londoño MC, Danger R, Giral M, Soulillou JP, Sánchez-Fueyo A, Brouard S. A need for biomarkers of operational tolerance in liver and kidney transplantation. Am J Transplant. 2012;12:1370–7. doi:10.1111/j.1600-6143.2012.04035.x.

Lorho R, Turlin B, Aqodad N, et al. C4d: a marker for hepatic transplant rejection. Transplant Proc. 2006;38:2333–4.

Lunz JG, Contrucci S, Ruppert K, et al. Replicative senescence of biliary epithelial cells precedes bile duct loss in chronic liver allograft rejection: increased expression of p21(WAF1/Cip1) as a disease marker and the influence of immunosuppressive drugs. Am J Pathol. 2001;158:1379–90.

Martínez-Llordella M, Puig-Pey I, Orlando G, et al. Multiparameter immune profiling of operational tolerance in liver transplantation. Am J Transplant. 2007;7:309–19.

Martínez-Llordella M, Lozano JJ, Puig-Pey I, et al. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. J Clin Invest. 2008;118:2845–57. doi:10.1172/JCI35342.

Massoud O, Heimbach J, Viker K, et al. Noninvasive diagnosis of acute cellular rejection in liver transplant recipients: a proteomic signature validated by enzyme-linked immunosorbent assay. Liver Transpl. 2011;17:723–32. doi:10.1002/lt.22266.

Mazariegos GV, Zahorchak AF, Reyes J, Chapman H, Zeevi A, Thomson AW. Dendritic cell subset ratio in tolerant, weaning and non-tolerant liver recipients is not affected by extent of immunosuppression. Am J Transplant. 2005;5:314–22.

Millán O, Rafael-Valdivia L, Torrademé E, et al. Intracellular IFN-γ and IL-2 expression monitoring as surrogate markers of the risk of acute rejection and personal drug response in de novo liver transplant recipients. Cytokine. 2013;61:556–64. doi:10.1016/j.cyto.2012.10.026.

Mimuro J, Mizuta K, Kawano Y, et al. Impact of acute cellular rejection on coagulation and fibrinolysis biomarkers within the immediate post-operative period in pediatric liver transplantation. Pediatr Transplant. 2010;14:369–76. doi:10.1111/j.1399-3046.2009.01248.x.

Minguela A, Miras M, Bermejo J, et al. HBV and HCV infections and acute rejection differentially modulate CD95 and CD28 expression on peripheral blood lymphocytes after liver transplantation. Hum Immunol. 2006;67:884–93.

Neuberger J. Incidence, timing, and risk factors for acute and chronic re-jection. Liver Transpl Surg. 1997;5:S30–6.

Neumann UP, Langrehr JM, Neuhaus P. Chronic rejection after human liver transplantation. Graft. 2002;5:102–7.

Ninova DI, Wiesner RH, Gores GJ, Harrison JM, Krom RA, Homburger HA. Soluble T lymphocyte markers in the diagnosis of cellular rejection and cytomegalovirus hepatitis in liver transplant recipients. J Hepatol. 1994;21:1080–5.

O'Leary JG, Kaneku H, Jennings LW, et al. Preformed class II donor-specific antibodies are associated with an increased risk of early rejection after liver transplantation. Liver Transpl. 2013;19:973–80. doi:10.1002/lt.23687.

O'Leary JG, Demetris AJ, Friedman LS, et al. The role of donor-specific HLA alloantibodies in liver transplantation. Am J Transplant. 2014a;14:779–87. doi:10.1111/ajt.12667.

O'Leary JG, Kaneku H, Demetris AJ, et al. Antibody-mediated rejection as a contributor to previously unexplained early liver allograft loss. Liver Transpl. 2014b;20:218–27. doi:10.1002/lt.23788.

O'Leary JG, Kaneku H, Banuelos N, Jennings LW, Klintmalm GB, Terasaki PI. Impact of IgG3 subclass and C1q-fixing donor-specific HLA alloantibodies on rejection and survival in liver transplantation. Am J Transplant. 2015;15:1003–13. doi:10.1111/ajt.13153.

Oetting WS, Guan W, Schladt DP, et al. Donor polymorphisms of TLR4 associated with graft failure in liver transplant recipients. Liver Transpl. 2012;18:1399–405. doi:10.1002/lt.23549.

Perkins JD, Nelson DL, Rakela J, Grambsch PM, Krom RA. Soluble interleukin-2 receptor level as an indicator of liver allograft rejection. Transplantation. 1989;47:77–81.

Platz KP, Mueller AR, Haller GW, et al. Determination of alpha- and Pi-glutathione-S-transferase will improve monitoring after liver transplantation. Transplant Proc. 1997;29:2827–9.

Pons JA, Revilla-Nuin B, Baroja-Mazo A, et al. FoxP3 in peripheral blood is associated with operational tolerance in liver transplant patients during immunosuppression withdrawal. Transplantation. 2008;86:1370–8. doi:10.1097/TP.0b013e318188d3e6.

Pons JA, Revilla-Nuin B, Ramírez P, Baroja-Mazo A, Parrilla P. Development of immune tolerance in liver transplantation. Gastroenterol Hepatol. 2011;34:155–69. doi:10.1016/j.gastrohep.2010.11.007.

Puig-Pey I, Bohne F, Benitez C, et al. Characterization of gammadelta T cell subsets in organ transplantation. Transpl Int. 2010;23:1045–55. doi:10.1111/j.1432-2277.2010.01095.x.

Raschzok N, Reutzel-Selke A, Schmuck RB, et al. CD44 and CXCL9 serum protein levels predict the risk of clinically significant allograft rejection after liver transplantation. Liver Transpl. 2015. doi:10.1002/lt.24164.

Ravaioli M, Neri F, Lazzarotto T, et al. Immunosuppression modifications based on an immune response assay: results of a randomized. Control Trial Transplant. 2015. doi:10.1097/TP.0000000000000650.

Reding R, Gras J, Truong DQ, Wieërs G, Latinne D. The immunological monitoring of alloreactive responses in liver transplant recipients: a review. Liver Transpl. 2006;12:373–83.

Rodrigo E, López-Hoyos M, Corral M, et al. ImmuKnow as a diagnostic tool for predicting infection and acute rejection in adult liver transplant recipients: a systematic review and meta-analysis. Liver Transpl. 2012;18:1245–53. doi:10.1002/lt.23497.

Rodríguez-Perálvarez M, Germani G, Tsochatzis E, et al. Predicting severity and clinical course of acute rejection after liver transplantation using blood eosinophil count. Transpl Int. 2012;25:555–63. doi:10.1111/j.1432-2277.2012.01457.x.

Sánchez-Fueyo A. Hot-topic debate on tolerance: immunosuppression withdrawal. Liver Transpl. 2011;17:S69–73. doi:10.1002/lt.22421.

Scornik JC, Soldevilla-Pico C, Van der Werf WJ, et al. Susceptibility of liver allografts to high or low concentrations of preformed antibodies as measured by flow cytometry. Am J Transplant. 2001;1:152–6.

Testro AG, Visvanathan K, Skinner N, et al. Acute allograft rejection in human liver transplant recipients is associated with signaling through toll-like receptor 4. J Gastroenterol Hepatol. 2011;26:155–63. doi:10.1111/j.1440-1746.2010.06324.x.

Thurairajah PH, Carbone M, Bridgestock H, et al. Late acute liver allograft rejection; a study of its natural history and graft survival in the current era. Transplantation. 2013;95:955–9. doi:10.1097/TP.0b013e3182845f6c.

Umeshita K, Monden M, Tono T, et al. Determination of the presence of interleukin-6 in bile after orthotopic liver transplantation. Its role in the diagnosis of acute rejection. Ann Surg. 1996;223:204–11.

Wang Y, Zhang M, Liu ZW, et al. The ratio of circulating regulatory T cells (Tregs)/Th17 cells is associated with acute allograft rejection in liver transplantation. PLoS One. 2014;9:e112135. doi:10.1371/journal.pone.0112135.

Warlé MC, Metselaar HJ, Hop WC, et al. Early differentiation between rejection and infection in liver transplant patients by serum and biliary cytokine patterns. Transplantation. 2003;75:146–51.

Wei L, Gong X, Martinez OM, Krams SM. Differential expression and functions of microRNAs in liver transplantation and potential use as non-invasive biomarkers. Transpl Immunol. 2013;29:123–9. doi:10.1016/j.trim.2013.08.005.

Wei W, Huang XH, Liang D, Zeng YY, et al. A proteomic analysis of transplanted liver in a rat model of chronic rejection. Clin Res Hepatol Gastroenterol. 2015;39:340–50. doi:10.1016/j.clinre.2014.10.005.

Yu X, Liu Z, Wang Y, et al. Characteristics of Vδ1(+) and Vδ2(+) γδ T cell subsets in acute liver allograft rejection. Transpl Immunol. 2013;29:118–22. doi:10.1016/j.trim.2013.09.001.

Yu X, Wei B, Dai Y, et al. Genetic polymorphism of interferon regulatory factor 5 (IRF5) correlates with allograft acute rejection of liver transplantation. PLoS One. 2014;9:e94426. doi:10.1371/journal.pone.0094426.

Biomarkers for Hepatocellular Carcinoma in East Asia

42

Peipei Song, Wei Tang, and Norihiro Kokudo

Contents

Abstract

Hepatocellular carcinoma (HCC) is undoubtedly a great health threat in East Asia, with the highest incidence of an age-standardized rate of 31.9 per 100,000 in men. In terms of the absolute number of cases, almost half a million cases were reported in China, Japan, and South Korea in 2012. Worldwide, alpha-fetoprotein (AFP) testing and abdominal ultrasound (US) every 6 months are recommended

P. Song (✉) • W. Tang (✉) • N. Kokudo (✉)
Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
e-mail: ppsong-tky@umin.ac.jp; TANG-SUR@h.u-tokyo.ac.jp; KOKUDO-2SU@h.u-tokyo.ac.jp

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_4

for routine surveillance of HCC in high-risk patients according to many HCC guidelines, and AFP has also been used as a diagnostic test for HCC and to evaluate prognosis and monitor recurrence following treatment. However, controversy regarding the clinical utility of AFP has arisen in the West and East in recent years. This controversy is also evident in HCC guidelines in countries in East Asia. Advances in technology and greater understanding of the pathology of HCC have led to the discovery of novel biomarkers. Data have indicated that the combined testing of AFP, the lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), or des-γ-carboxyprothrombin (DCP) could help to increase the sensitivity of diagnosis of HCC, but this approach is currently used in only a few countries, such as Japan. In recent years, numerous studies have investigated the clinical usefulness of some novel biomarkers in early diagnosis of HCC, including Dickkopf-1 (DKK1), midkine (MDK), and microRNA (miRNA). Moreover, the prognostic significance of some biomarkers, such as miRNA, gamma-glutamyl transferase (GGT), and indocyanine green retention 15 min after administration (ICG-R15), has also been evaluated. However, further studies are needed to better characterize the accuracy and potential role of these approaches in clinical practice. The prevailing hope is that novel biomarkers can support clinicians in their daily practice and improve care for patients with HCC.

Keywords

HCC • Biomarker • Guideline • Surveillance • Diagnosis • Prognosis

List of Abbreviations

AASLD	American Association for the Study of Liver Disease
AFP	Alpha-fetoprotein
AFP-L3	The lens culinaris agglutinin-reactive fraction of AFP
ALD	Alcohol-induced liver disease
APASL	Asian Pacific Association for the Study of the Liver
CI	Confidence interval
CT	Computed tomography
DCP	Des-γ-carboxyprothrombin
DKK1	Dickkopf-1
EASL	European Association for the Study of the Liver
EBM	Evidence-based medicine
Gd-EOB-DTPA	Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid
GGT	Gamma-glutamyl transferase
GP73	Golgi protein 73
GPC3	Glypican-3
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HR	Hazard ratio
ICG-R15	Indocyanine green retention 15 min after administration
IL-6	Interleukin-6

INASL	Indian National Association for Study of the Liver
KLCSG	Korean Liver Cancer Study Group
LR+	Positive likelihood ratio
MDK	Midkine
miRNA	MicroRNA
MRI	Magnetic resonance imaging
NAFLD	Nonalcoholic fatty liver disease
NCC	National Cancer Center
NCCN	National Comprehensive Cancer Network
NHFPC	National Health and Family Planning Commission
OR	Odds ratio
SCCA	Squamous cell carcinoma antigen
TACE	Transarterial chemoembolization
TARE	Transarterial radioembolization
US	Ultrasound
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Key Facts on Multiple Biomarkers for HCC Surveillance and Diagnosis

- Data have indicated that the combined testing of DCP and AFP or AFP-L3 could help to increase the sensitivity of diagnosis of HCC. The combined testing of two biomarkers had an OR of 6.29–59.81 in diagnosing HCC smaller than 5 cm in diameter, which was better than that of one biomarker alone.
- However, the combined testing of DCP and AFP or AFP-L3 is used in only a few countries, such as Japan. Although the clinical usefulness of combined testing of DCP and AFP or AFP-L3 has also been noted by several retrospective studies published in South Korea and China, it has not been recommended by HCC guidelines in South Korea and China until now.
- Data showed that the measurement of DKK1 and AFP together improved the accuracy with which HCC was diagnosed in comparison to any single test alone.
- Data showed that serum MDK had a markedly higher level of sensitivity than AFP (86.9% vs. 51.9%) but a similar level of specificity (83.9% vs. 86.3%). MDK has a significantly higher level of sensitivity than AFP (80% vs. 40%) at diagnosing very early stage HCC.
- Data showed that the combination of miRNA-21 with AFP improved the power of differentiation between HCC and chronic hepatitis, with a sensitivity of 81.0% and a specificity of 80%.

The above is a list of key facts regarding the current status of multiple biomarkers for HCC surveillance and diagnosis, including the combined testing of DCP and AFP or AFP-L3, the combined testing of DKK1 and AFP, the combined testing of MDK and AFP, and the combined testing of miRNA-21 with AFP.

Key Facts on Alpha-Fetoprotein (AFP)

- Serum AFP has traditionally and widely been used as a tumor marker of HCC over the past two decades.
- Elevated serum AFP and a typical enhancement pattern in dynamic imaging have provided critical clues for the diagnosis of HCC.
- However, based on the high accuracy of up-to-date radiologic modalities, the importance in AFP has diminished in recent guidelines for diagnosis of HCC.
- AFP has been excluded from the surveillance criteria in the HCC guidelines published by the AASLD in 2010, and AFP is regarded as a suboptimal tool for surveillance according to the HCC guidelines published by the EASL in 2012. It is, however, still recommended by many HCC guidelines in Asia, such as guidelines in Japan, and China.
- AFP was one of the most robust predictors of death in patients with cirrhosis and HCC, and it also has significance at predicting survival after liver transplantation.
- A change in AFP levels has been found to correlate with radiologic response and overall survival after locoregional therapy, such as transarterial chemoembolization (TACE), transarterial radioembolization (TARE).

The above is a list of key factors regarding the current status of using AFP for HCC surveillance, diagnosis, and prognosis as well as controversies over that use in the West and East.

Definitions of Words and Terms

Alpha-fetoprotein (AFP)	A host cellular protein. High levels can occur in persons with HCC.
Chronic HBV infection	Persistence of hepatitis B surface antigen (HBsAg) for 6 months or more after acute infection with HBV.
Chronic HCV infection	Continued presence of HCV 6 months or more after acquiring infection.
Cirrhosis	An advanced stage of liver disease characterized by extensive hepatic fibrosis, nodularity of the liver, alteration of liver architecture, and disrupted hepatic circulation.
Des-gamma carboxyprothrombin (DCP)	Also known as protein induced by vitamin K absence/antagonist-II (PIVKA-II), it is an abnormal prothrombin that lacks carboxylation of specific amino-terminal glutamic acid residues.
Gamma-glutamyl transferase (GGT)	An enzyme catalyzing hydrolysis of glutathione and transfer of gamma-glutamyl residue; a high prevalence of abnormal GGT in patients with primary or

	secondary liver cancer was showed by many clinical studies.
Guideline	The standardized management of care that specifies appropriate diagnoses and treatments based on scientific research evidence and collaborations between medical professionals involved in the treatment of a given condition.
Hepatocellular carcinoma (HCC)	Primary cancer of the liver arising in hepatocytes.
Sensitivity of a test	The ability of a test to correctly identify those with the infection or disease (i.e., true positives/true positives + false negatives).
Specificity of a test	The ability of a test to correctly identify those without the infection or disease (i.e., true negatives/true negatives + false positives).

Introduction

According to data from the World Health Organization (WHO), there were 14.1 million new cancer cases worldwide in 2012, 8.2 million deaths due to cancer, and 32.6 million people living with cancer (WHO 2012). Liver cancer is the fifth most common cancer in men (554,000 cases, 7.5% of the total) and the ninth in women (228,000 cases, 3.4% of the total) (WHO 2012). Hepatocellular carcinoma (HCC) accounts for more than 90% of primary liver cancers and is a major global health problem due to the high prevalence of infection with the hepatitis B virus (HBV) and/or hepatitis C virus (HCV) as risk factors. Over the past two decades, the clinical care for patients with HBV- or HCV-related liver disease has advanced considerably due to developments in diagnostic procedures and improvements in therapy and prevention (Zhang et al. 2013; Shaheen and Idrees 2015). However, the incidence of HCC worldwide is increasing, and this is likely to be associated with the often prolonged period between viral infection and the manifestation of HCC. Moreover, evidence has shown that surgical resection and liver transplantation may offer the best potential for treating HCC but are only available to patients whose tumors are detected early. The overall 5-year survival rate is 40%, but liver resection to treat early HCC could lead to a 5-year survival rate of 60–70% (Gao et al. 2012; Llovet and Bruix 2008). Thus, strategies to surveil and diagnose HCC at an earlier stage are urgently needed when curable interventions can be offered to achieve long-term disease-free survival for patients with HCC.

Serum biomarkers are striking potential tools for surveillance and early diagnosis of HCC thanks to the noninvasive, objective, and reproducible assessments they potentially enable. Worldwide, alpha-fetoprotein (AFP) testing and abdominal ultrasound (US) every 6 months are recommended for routine surveillance of HCC in

high-risk patients according to many HCC guidelines (Song et al. 2012), and AFP has also been used as a diagnostic test for HCC and to evaluate prognosis and monitor recurrence following treatment (Rich and Singal 2014). However, controversy regarding the clinical utility of AFP has arisen in the West and East in recent years. The same controversy is also evident in HCC guidelines in countries in East Asia. Other biomarkers including the lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), des-γ-carboxyprothrombin (DCP), Dickkopf-1 (DKK1), midkine (MDK), microRNA (miRNA), gamma-glutamyl transferase (GGT), indocyanine green retention 15 min after administration (ICG-R15), Golgi protein 73 (GP73), interleukin-6 (IL-6), squamous cell carcinoma antigen (SCCA), glypican-3 (GPC3), osteopontin, and vascular endothelial growth factor (VEGF) are being studied in this regard. Furthermore, increasing attention has focused on the clinical utility of biomarkers as pretreatment predictors for tumor recurrence and as posttreatment monitors.

Clinical Practice Guidelines for HCC in East Asia

Asian countries report approximately three-fourths of the cases of HCC worldwide. East Asia is the region with the highest incidence, with an age-standardized rate of 31.9 per 100,000 in men (WHO 2012). In terms of absolute numbers of cases, almost half a million cases were reported in China, Japan, and South Korea in 2012. Of particular note is the fact that there are approximately 395,000 cases of HCC in China, which accounts for 50% of HCC cases worldwide (WHO 2012).

Currently, the overall prevalence of HCC in China is 26–32 per 100,000 persons, and in some areas prevalence can be as high as 70–80 per 100,000 (Song et al. 2013b; Yuen et al. 2009). HCC is now the second most common cancer in urban areas and the first most common in rural areas. HCC ranks as the second leading cause of cancer-related deaths in males and the third leading cause of cancer-related deaths in females, with a total mortality rate of 26.26 per 100,000 in China (Song et al. 2013a; Song 2013). In Japan, HCC ranks as the third leading cause of cancer-related deaths in males and the fifth leading cause of cancer-related deaths in females, with more than 30,000 patients dying of HCC every year (Ikai et al. 2007; Chung et al. 2010). In South Korea, statistics published by the central cancer registry in 2013 indicated that there are 12,189 cases involving males and 4,274 cases involving females, making HCC the fourth most common cancer in males and the sixth most common cancer in females (Korean Liver Cancer Study and National Cancer Center 2015a). HCC is the top-ranked cause of death in people in their 40s and 50s, with 22.5 people (male, 33.7; female, 11.3) per 100,000 population dying annually from HCC (Korean Liver Cancer Study and National Cancer Center 2015b).

HCC is undoubtedly a great health threat in East Asia. Coping with HCC is a challenge not only for clinicians but also for health policymakers. With the development of evidence-based medicine (EBM), the concept of incorporating "current best evidence into clinical decision-making" has garnered substantial attention

worldwide. Guided by current best evidence, many clinical practice guidelines for HCC have been published worldwide (Song et al. 2014b). In East Asian countries, the Korean Liver Cancer Study Group (KLCSG) and National Cancer Center (NCC) jointly published clinical practice guideline for HCC (KLCSG-NCC Korea Guideline) in 2003 (Park et al. 2004), revised it in 2009 (Korean Liver Cancer Study and National Cancer Center 2009), and then updated it in 2014 (Korean Liver Cancer Study and National Cancer Center 2015a). With the support of the Japanese Ministry of Health, Labor, and Welfare, the first evidence-based clinical practice guideline for HCC in Japan (J-HCC Guideline) was published in 2005 (Makuuchi and Kokudo 2006), revised in 2009 (2010), and then updated in 2013 (Japan Society of Hepatology 2013). In China, "The Expert Consensus on the Treatment Standards for Hepatocellular Carcinoma (Chinese HCC Consensus)" was published in 2009 based on the consensus opinions of more than 60 experts (Chinese Anti-Cancer Association Society of Liver Cancer 2009), and the National Health and Family Planning Commission (NHFPC) of the People's Republic of China also published the updated "Guideline on Diagnosis and Treatment for Primary Liver Cancer" (NHFPC HCC Guideline) in 2011 (National Health and Family Planning Commission 2011) to promote the standardized management of HCC.

Biomarkers for HCC Surveillance and Diagnosis in Japan, China, and South Korea

Several cohort studies have documented the cost-effectiveness and survival benefit of surveillance programs to detect HCC early (Yang et al. 2011; Stravitz et al. 2008). Worldwide, many guidelines for HCC management recommend HCC surveillance, including the guidelines established by the American Association for the Study of Liver Disease (AASLD), the National Comprehensive Cancer Network (NCCN), and the Asian Pacific Association for the Study of the Liver (APASL).

The Current Status of Surveillance and Early Diagnosis of HCC in Japan

Approximately three-fourths of cases of HCC worldwide occurred in Asian countries due to the high prevalence of chronic infection with HBV (Yuen et al. 2009). However, chronic hepatitis C is more commonly related to liver cancer in Japan, which accounts for up to 70% of these cases (Chung et al. 2010).

In Japan, where HCV is the most significant etiological factor for developing HCC, there is a more detailed definition for high-risk patients of HCC (Table 1) – the "very-high-risk group" includes patients with HBV- or HCV-related liver cirrhosis, and the "high-risk group" includes patients with HBV- or HCV-related chronic liver disease or liver cirrhosis due to other causes.

Table 1 The epidemiology, surveillance, and early diagnosis of HCC in Japan

Items	Current status
Epidemiology	The third leading cause of cancer-related deaths in males and the fifth in females
Etiological factors	70% of patients with an HCV infection, 15–20% of patients with an HBV infection
Major at-risk population	High-risk group: patients with an HBV/HCV infection or cirrhosis due to other causes; Very-high-risk group: patients with HBV-/HCV-related cirrhosis
Guideline	J-HCC Guideline published in 2005, revised in 2009, updated in 2013
Surveillance tools	US and combined test of DCP and AFP or AFP-L3
Surveillance criteria	3–4 months for the very-high-risk group; 6-month intervals for the high-risk group
Diagnosis	Dynamic CT/MRI for definitive diagnosis; DCP/AFP/AFP-L3 for adjunctive diagnosis

Currently, AFP, AFP-L3, and DCP are widely and routinely used for HCC surveillance in Japan, and these tests are covered by Japan's national health insurance as serological biomarkers for HCC surveillance in clinical settings. According to the criteria for HCC surveillance and diagnosis in the latest version of the J-HCC Guideline published in 2013 (Fig. 1), the combined testing of AFP, AFP-L3, or DCP and US should be performed at intervals of 3–4 months for the very-high-risk group and at intervals of 6 months for the high-risk group.

If US suggests a new nodular lesion in HCC surveillance in Japan, dynamic computed tomography (CT) or dynamic magnetic resonance imaging (MRI) will be performed to make a differential diagnosis; if the AFP level continues to rise or has increased to 200 ng/ml or more, the DCP level is at least 40 mAU/ml, or the AFP-L3 fraction is 15% or more, dynamic CT/MRI will be considered, even if US shows no evidence of a tumor (Japan Society of Hepatology 2013).

In 2009, 200 Japanese experts were surveyed regarding the use of biomarkers in HCC surveillance in clinical practice in Japan, and responses indicated that 72% of these experts simultaneously measured the biomarkers of AFP, AFP-L3, and DCP, and 44% of the experts combined these measurements with US (Kudo 2010). Since most high-risk patients were closely followed before developing HCC, HCC nodules were detected in the early stage in more than 60% of patients in Japan (Song et al. 2013c).

The Current Status of Surveillance and Early Diagnosis of HCC in China

In China, HBV is the biggest factor for developing HCC; approximately 85% of Chinese cases of HCC are HBV-related, 10% of cases are HCV-related, and some cases involve HBV and HCV superinfection (Tanaka et al. 2011). The high-risk

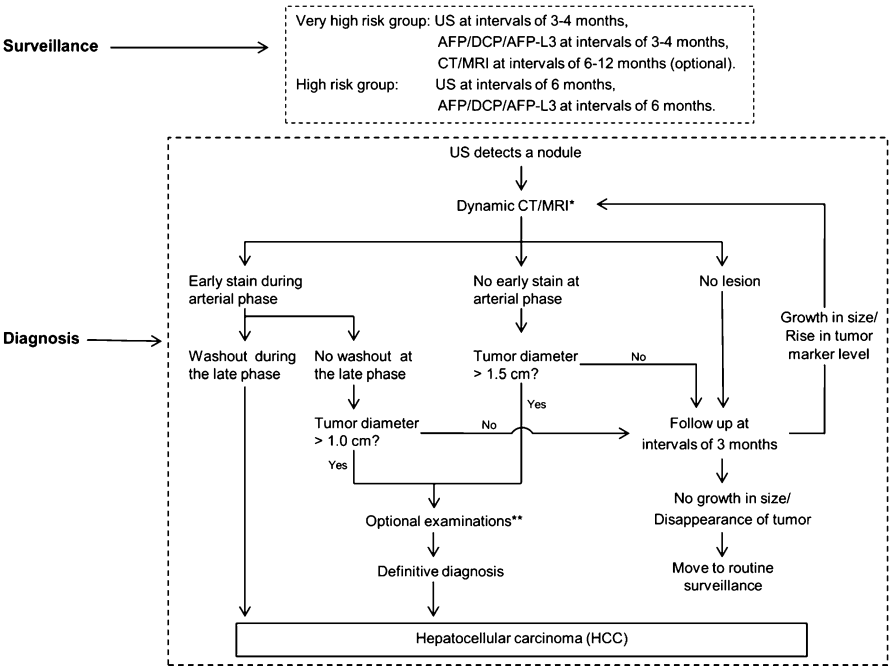

Fig. 1 Surveillance and diagnostic algorithm for clinical management of HCC according to the latest J-HCC Guidelines published in 2013 in Japan. *Note: If the AFP level rises continuously or has increased to 200 ng/ml or more, the DCP level is at least 40 mAU/ml, or the AFP-L3 fraction is 15% or more, dynamic CT/MRI will be considered, even if US shows no evidence of a tumor. **CT-angiography/liver-specific contrast-enhanced MRI/contrasonography/liver biopsy

group for HCC in China includes patients chronically infected with HBV, HCV, or HBV and HCV superinfection, and patients with cirrhosis, alcoholism, diabetes mellitus, or a family history of HCC. For patients aged 35–40 years, AFP and US should be performed every 6 months according to the Chinese Guideline (Chinese Anti-Cancer Association Society of Liver Cancer 2009, National Health and Family Planning Commission 2011) (Table 2). If the AFP level continues to rise or US suggests a new nodular lesion, a differential diagnosis will be made based on diagnostic imaging, serological diagnosis, or histological diagnosis (Fig. 2).

At present, AFP measurement and US at 6-month intervals are the standard tools for HCC surveillance in China. AFP is considered to be a useful and feasible tool for HCC surveillance and early diagnosis in China due to its convenience and particularly because of the fact that more than 60% of patients with HCC have an AFP level of >400 ng/ml (Song et al. 2012). The clinical usefulness of AFP in China has been confirmed by a randomized controlled trial involving 18,816 patients ages 35–59 years with an HBV infection or a history of chronic hepatitis. The patients were randomly assigned to a screening (9,373) or control (9,443) group undergoing AFP measurement and US every 6 months. Results showed that biannual screening

Table 2 The epidemiology, surveillance, and early diagnosis of HCC in China

Items	Current status
Epidemiology	The second leading cause of cancer-related deaths in males and the third in females
Etiological factors	85% of patients with an HBV infection, 10% of patients with an HCV infection
Major at-risk population	People with an HBV infection; 93 million HBV carriers, 20 million people with a chronic HBV infection
Guideline	Chinese HCC Consensus published in 2009; NHFPC HCC Guideline updated in 2011
Surveillance tools	AFP and US
Surveillance criteria	6-month interval for HCC the high-risk population ages 35–40
Diagnosis	US/CT/MRI and biopsy for differential diagnosis; AFP for adjunctive diagnosis

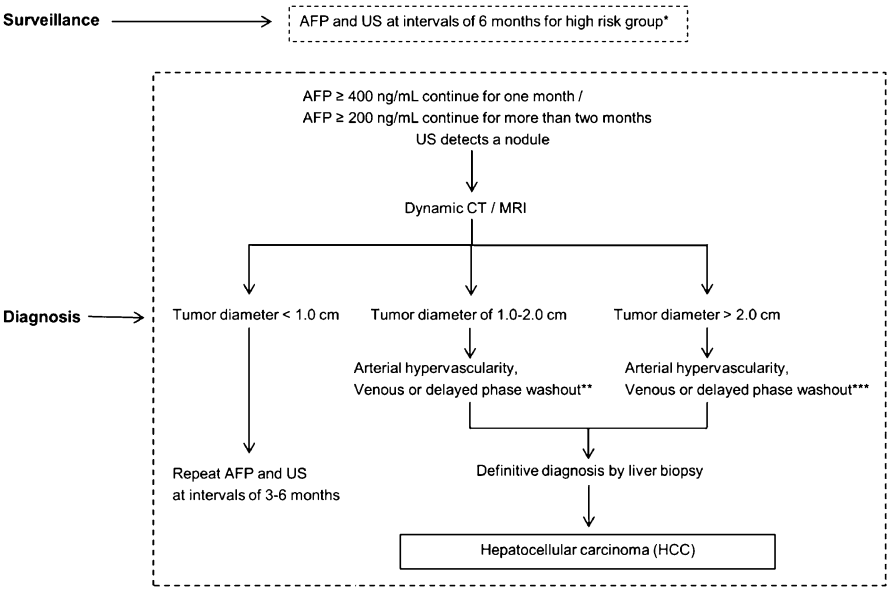

Fig. 2 Surveillance and diagnostic algorithm for clinical management of HCC according to the latest NHFPC HCC Guideline published in 2011 in China. NHFPC, the National Health and Family Planning Commission of the People's Republic of China. *Evidences show liver cirrhosis, HBV and/or HCV infection. ** Detected by dynamic CT and MRI. ***Detected by dynamic CT or MRI

with AFP and US significantly reduced mortality. Screened patients had a survival rate of 65.9% at 1 year, 52.6% at 3 years, and 46.4% at 5 years versus unscreened patients who had a survival rate of 31.2% at 1 year, 7.2% at 3 years, and 0% at 5 years (Zhang et al. 2004).

A study was conducted in China in 2002 to determine DCP and AFP levels in 60 patients with HCC and 30 patients with cirrhosis but no HCC in order to assess the value of DCP in surveilling and diagnosing Chinese patients with HCC (Cui et al. 2002). This study found no significant correlation between serum levels of DCP and AFP in the 60 patients with HCC ($r_s = 1.101$, $p = 0.247$). DCP had a sensitivity of about 51.7% and a specificity of about 86.7%, while the combined tests of DCP and AFP had a sensitivity of 78.3%, which is higher than that of DCP (51.7%) or AFP (56.7%) alone. Another study assessed the clinical usefulness of DCP in Chinese patients with HCC in 2003 (Cui et al. 2003). This study involved 120 patients with HCC and 90 patients with cirrhosis. The study found no significant correlation between serum levels of DCP and AFP in the 120 patients with HCC ($r_s = 1.106$, $p = 0.249$). DCP had a sensitivity of 53.3% and a specificity of 85.6%, while the combined tests of DCP and AFP had a sensitivity of 78.3%, which is higher than that of DCP (53.3%) or AFP (58.3%) alone. In addition, many other studies have investigated the clinical utility of DCP for Chinese patients with HCC in recent years.

In 2014, a large-scale, multicenter study investigated the measurement of both AFP and DCP in differentiating Chinese patients with HCC (71.18% with an HBV infection) from patients without HCC and normal subjects. Results showed that the combined testing of DCP with a cut-off value of 86 mAU/mL and AFP with a cut-off value of 21 ng/mL resulted in a sensitivity of approximately 90% in diagnosis of HCC, which was significantly higher than that for DCP or AFP alone, and this finding held even for a tumor smaller than 2.0 cm (Song et al. 2014a). These results suggest that the measurement of both AFP and DCP may facilitate the diagnosis of patients with a broad range of HCC. However, the clinical utility of DCP in China has not been noted in Chinese guidelines on HCC, and more large-scale prospective studies should be performed to provide sufficient evidence.

The Current Status of Surveillance and Early Diagnosis of HCC in South Korea

In South Korea, HBV is the biggest factor for developing HCC. One study of patients with HCC reported that underlying liver diseases included hepatitis B (72.3%), hepatitis C (11.6%), alcoholic liver disease (10.4%), and non-B non-C hepatitis (0.7%) (Korean Liver Cancer Study and National Cancer Center 2015b). Another study reported that 74.6% of HCC patients were positive for HBV, 9.3% were positive for HCV, 7.4% were long-term alcohol abusers, and 8.7% had unidentified causes (probably metabolic liver disease) (Kwak et al. 2014). HCC develops in 1–4% of cirrhotic patients annually and eventually develops in approximately one-third of cirrhotic patients (Ioannou et al. 2007).

According to the KLCSG-NCC Korea Guideline published in 2014, US was recommended for surveillance of patients with HBV/HCV or cirrhosis in the high-risk group (Table 3), and HCC is diagnosed on the basis of either pathology or clinical criteria ascertained with imaging techniques. When HCC is suspected in the

Table 3 The epidemiology, surveillance, and early diagnosis of HCC in South Korea

Items	Current status
Epidemiology	The top-ranked cause of death in people in their 40s and 50s; 22.5 people (male, 33.7; female, 11.3) per 100,000 population die annually from HCC
Etiological factors	70–75% of patients with an HBV infection, 10% of patients with an HCV infection
Major at-risk population	Patients with an HBV/HCV infection or cirrhosis due to other causes
Guideline	KLCSG-NCC Korea Guideline published in 2003, revised in 2009, updated in 2014
Surveillance tools	US
Surveillance criteria	6-month interval for HCC the high-risk population
Diagnosis	Dynamic contrast-enhanced CT/MRI or liver-specific contrast-enhanced MRI; Pathology

high-risk group during surveillance with US, dynamic contrast-enhanced CT/MRI or liver-specific contrast-enhanced MRI should be performed for diagnosis (Fig. 3). HCC can be diagnosed in the high-risk group if one or two of the aforementioned imaging techniques indicates that nodules $\geq$ 1 cm in diameter have typical features of HCC (including arterial phase enhancement with washout in the portal or delayed phase). Two or more imaging modalities are required to diagnose nodules 1–2 cm in diameter if a suboptimal imaging technique is used. Nodules $<$ 1 cm in diameter can be diagnosed as HCC in high-risk patients when both of the following conditions are met: typical features of HCC in two or more of the aforementioned imaging modalities and a continuous rise in serum AFP with hepatitis activity under control (Korean Liver Cancer Study and National Cancer Center 2015a).

In South Korea, AFP was not recommended for HCC surveillance but was regarded as an adjunctive diagnostic tool. Over the past 10 years, the utility of AFP has been described differently in the diagnostic criteria in three versions of the KLCSG-NCC Korea Guidelines. In the KLCSG-NCC Korea Guideline published in 2003 (Park et al. 2004), HCC was diagnosed based on imaging and AFP (AFP levels $\geq$400 ng/mL with one typical dynamic imaging technique, or AFP levels $<$400 ng/mL with two typical dynamic imaging techniques), regardless of tumor size. However, the revised KLCSG-NCC Korea Guideline published in 2009 (Korean Liver Cancer Study and National Cancer Center 2009) suggested that a tumor of 2 cm or larger in patients with liver cirrhosis that had characteristics typical of HCC in dynamic contrast enhancement CT or MRI could be diagnosed as HCC regardless of the serum AFP levels. If nodules in high-risk patients are smaller than 1 cm and diagnosis cannot be verified by a radiologic or histologic examination, a tumor marker test and US should be performed several times at an interval of 3–6 months to monitor for any increase in the size and level of tumor markers. In the latest version of the KLCSG-NCC Korea Guideline published in 2014 (Korean Liver Cancer Study and National Cancer Center 2015a), AFP was also recommended as an adjunctive diagnosis tool with two positive techniques of

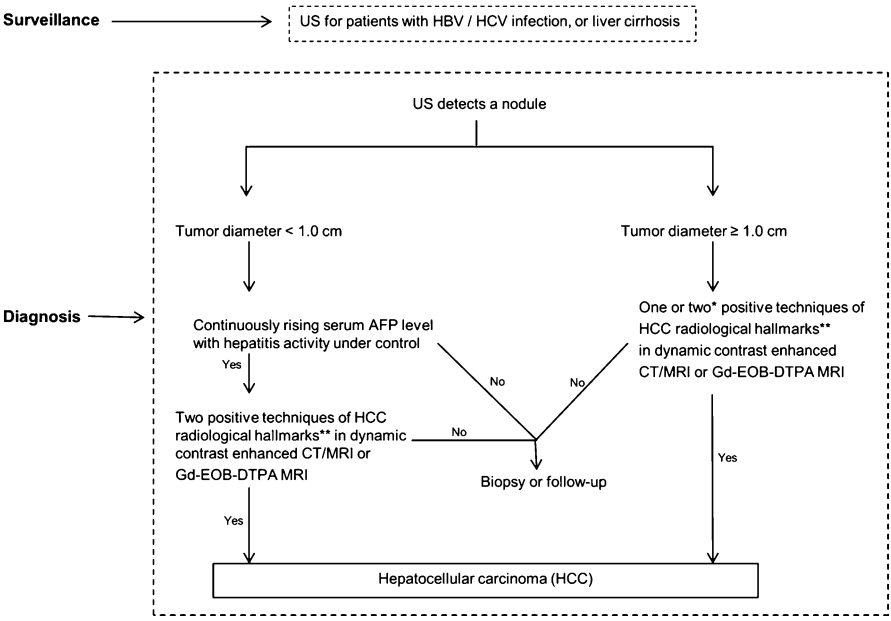

Fig. 3 Surveillance and diagnostic algorithm for clinical management of HCC according to the latest KLCSG-NCC Korea Guideline published in 2014 in South Korea. KLCSG, the Korean Liver Cancer Study Group; NCC, the National Cancer Center. *For diagnosis of nodules 1.0–2.0 cm in diameter, two or more imaging modalities are required if suboptimal imaging technique is used. **HCC radiological hallmarks include arterial phase enhancement with washout in portal or delayed phase

HCC radiological hallmarks in dynamic contrast-enhanced CT/MRI or gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) MRI for cases with tumor < 1 cm.

Like in Japan, use of DCP has also been approved in South Korea. Several Korean retrospective studies also reported the clinical usefulness of combined testing of the tumor markers AFP, AFP-L3, and DCP (Kim et al. 2006; Yoon et al. 2002). However, further well-designed studies are warranted to confirm the role of these markers in the diagnosis of HCC.

Controversies Regarding Use of Biomarkers in HCC Surveillance and Diagnosis in the East and West

AFP as a Traditional Biomarker for HCC Surveillance and Diagnosis

Serum AFP has traditionally and widely been used as a tumor marker of HCC over the past two decades. However, there is increasing debate regarding the utility of AFP as a surveillance test. An analysis of recent studies has indicated that the serum

AFP level is normal in up to 35% of cases of small HCC and can be nonspecifically elevated in patients with active hepatitis or active hepatocyte regeneration (Korean Liver Cancer Study and National Cancer Center 2015b). Elevated AFP levels may also be seen in patients with cirrhosis or exacerbation of chronic hepatitis or cholangiocarcinoma (Bertino et al. 2012; Nguyen et al. 2002).

Given these findings, US is regarded as a more appropriate test for surveillance with an acceptable diagnostic accuracy (sensitivity ranging from 58% to 89%, and specificity greater than 90%) (Singal et al. 2009; Bolondi 2003). Currently, US is recommended as the only tool for HCC surveillance in some Western countries. AFP has been excluded from the surveillance criteria in the HCC guidelines published by the AASLD in 2010 (Bruix et al. 2011), and AFP is regarded as a suboptimal tool for surveillance according to the HCC guidelines published by the European Association for the Study of the Liver (EASL) in 2012 (European Association for the Study of the Liver et al. 2012). Nevertheless, the results of US in the early detection of HCC are highly dependent on the expertise of the examiner and the quality of the equipment. Currently, the combination of AFP and US at approximately 6-month intervals is still recommended by many HCC guidelines in Asia, such as guidelines in Japan (Japan Society of Hepatology 2013), China (National Health and Family Planning Commission 2011), and guidelines published by the APASL (Omata et al. 2010). Thus, whether AFP should be excluded from surveillance criteria needs to be investigated in more large, randomized controlled trials.

Over the past few decades, elevated serum AFP and a typical enhancement pattern in dynamic imaging have provided critical clues for the diagnosis of HCC. AFP was recommended as an adjunctive diagnostic tool in HCC guidelines published in Western countries, including the guideline published by the EASL in 2000, that published by the AASLD in 2005, and that published by the NCCN in 2009. Nevertheless, the importance in AFP has diminished in recent guidelines for diagnosis of HCC and the importance of imaging has increased based on the high accuracy of up-to-date radiologic modalities.

According to updated HCC guidelines published by the AASLD in 2010 (Bruix et al. 2011), nodules larger than 1 cm found during US surveillance of a cirrhotic liver should be investigated further with either a 4-phase multidetector CT scan or dynamic contrast-enhanced MRI. If the appearance of the nodule is typical of HCC, the lesion should be treated as HCC; if the findings are not characteristic or the vascular profile is not typical, a second contrast-enhanced study involving another imaging modality should be performed, or the lesion should be biopsied. In agreement with updated guidelines from the AASLD, the panel that drafted the HCC guidelines of the NCCN in 2014 (Benson et al. 2014) also considered an imaging finding of classic enhancement to be more definitive in this instance since the level of serum AFP may be elevated in persons with certain nonmalignant conditions or it may be within normal limits in a substantial percentage of patients with HCC.

In Asian countries, according to the HCC guidelines published by the APASL in 2010 (Omata et al. 2010), typical HCC can be diagnosed based on imaging

regardless of tumor size if a typical vascular pattern, i.e., arterial enhancement with portal venous washout, is obtained on dynamic CT/MRI or contrast-enhanced US, AFP was recommended as an adjunctive diagnostic tool and AFP alone was not recommended for diagnosis of HCC. Similar recommendations were made by HCC guidelines published in Japan (Japan Society of Hepatology 2013), China (National Health and Family Planning Commission 2011), and South Korea (Korean Liver Cancer Study and National Cancer Center 2015a).

A continuously rising level of AFP or a level of 200 ng/mL or more was recommended as the cut-off value for AFP according to the versions of the J-HCC Guideline published in 2005, 2009, and 2013 (Japan Society of Hepatology 2013; Makuuchi and Kokudo 2006; The Japan Society of Hepatology 2010). A cut-off value of AFP $\geq$ 400 ng/mL was recommended by the Chinese HCC Consensus published in 2009 (Chinese Anti-Cancer Association Society of Liver Cancer 2009). A cut-off value of AFP $\geq$ 400 ng/mL for more than 1 month or AFP $\geq$ 200 ng/mL for more than 2 months was recommended by the NHFPC HCC Guideline updated in 2011 (National Health and Family Planning Commission 2011). The 2003 version of the KLCSG-NCC Korea Guideline published in South Korea recommended a cut-off value of AFP $\geq$ 400 ng/mL (Park et al. 2004), while the 2009 version recommended a cut-off value of AFP $\geq$ 200 ng/mL (Korean Liver Cancer Study and National Cancer Center 2009). When a tumor $\geq$ 2 cm in patients with liver cirrhosis has typical characteristics of HCC in dynamic contrast enhancement CT or MRI, it can be diagnosed as HCC regardless of the serum AFP levels. The latest version of the KLCSG-NCC Korea Guideline published in 2014 (Korean Liver Cancer Study and National Cancer Center 2015a) recommends a continuous rise in serum AFP with hepatitis activity under control as an adjunctive diagnostic tool when a nodule $<$ 1 cm in diameter has typical features of HCC according to two or more dynamic imaging modalities.

The Combined Testing of AFP, AFP-L3, and DCP for HCC Surveillance and Diagnosis

Current expert opinion from Western countries has been rather critical of the clinical value of biomarkers. Imaging-based surveillance criteria were recommended by guidelines from Western countries, such as the updated HCC guidelines published by the AASLD in 2000 (Bruix et al. 2011) and similar guidelines published by the EASL in 2012 (European Association for the Study of the Liver et al. 2012). In Asian countries, as typified by Japan's HCC guidelines (Japan Society of Hepatology 2013), US and measurement of AFP, AFP-L3, or DCP should be performed at intervals of 3–4 months for the very-high-risk group (patients with HBV- or HCV-related liver cirrhosis) and at intervals of 6 months for the high-risk group (patients with HBV- or HCV-related chronic liver disease or liver cirrhosis due to other causes). The testing of AFP, AFP-L3, and DCP levels is covered by Japan's national health insurance as serological biomarkers for HCC surveillance in clinical settings.

When diagnosing HCC in cases of a tumor smaller than 5 cm in diameter (Tateishi et al. 2008), AFP with a cut-off value of 20 ng/mL had a sensitivity of 49–71%, a specificity of 49–86%, an odds ratio (OR) of 4.06, and a positive likelihood ratio (LR+) of 2.45. AFP with a cut-off value of 200 ng/mL had a sensitivity of 8–32%, a specificity of 76–100%, an OR of 6.99, and an LR+ of 5.85. AFP-L3 can differentiate an increase in AFP due to HCC from that due to benign liver disease. AFP-L3 with a cut-off value of 10% had a sensitivity of 22–33%, a specificity of 93–99%, an OR of 6.43, and an LR+ of 4.89 in diagnosing HCC smaller than 5 cm in diameter. AFP-L3 with a cut-off value of 15% had a sensitivity of 21–49%, a specificity of 94–100%, an OR of 10.50, and an LR+ of 13.10. DCP has also been recognized as a highly specific marker for HCC. DCP with a cut-off value of 40 mAU/mL had a sensitivity of 14–54%, a specificity of 95–99%, an OR of 21.31, and an LR+ of 12.60 in diagnosing HCC smaller than 5 cm in diameter. DCP with a cut-off value of 100 mAU/mL had a sensitivity of 7–56%, a specificity of 72–100%, an OR of 6.70, and an LR+ of 4.91.

Data have indicated that the combined testing of DCP and AFP or AFP-L3 could help to increase the sensitivity of diagnosis of HCC. The combined testing of two biomarkers had a OR of 6.29–59.81 in diagnosing HCC smaller than 5 cm in diameter (Tateishi et al. 2008), which was better than that of one biomarker alone. However, the combined testing of DCP and AFP or AFP-L3 is used in only a few countries, such as Japan. Although the clinical usefulness of combined testing of DCP and AFP or AFP-L3 has also been noted by several retrospective studies published in South Korea and China, this testing has not been recommended by HCC guidelines in South Korea and China until now. Further well-designed studies are warranted to confirm the roles of these biomarkers in the diagnosis of HCC.

The Clinical Utility of AFP, AFP-L3, and DCP in HCC Prognosis

The biomarkers AFP, AFP-L3, and DCP have been evaluated for their power in diagnosing HCC, and they have also been studied for their prognostic significance. A high level of AFP expression in serum correlates with profound cell proliferation, profound angiogenesis, and limited apoptosis and is associated with a poor prognosis (Mitsuhashi et al. 2008; Llovet et al. 2012). AFP was one of the most robust predictors of death in patients with cirrhosis and HCC (Tandon and Garcia-Tsao 2009), and it also has significance at predicting survival after liver transplantation (Mailey et al. 2011). Changes in AFP while on the waitlist also predicted posttransplant survival, and identifying these changes could facilitate better patient selection to optimize organ allocation and posttransplant outcomes (Rich and Singal 2014). A change in AFP levels has been found to correlate with radiologic response and overall survival after locoregional therapy. As an example, a 50% decrease in AFP levels resulted in a better time-to-progression [hazard ratio (HR): 2.8, 95% confidence interval (CI): 1.5–5.1] and overall survival (HR: 2.7, 95% CI: 1.6–4.6) in

comparison to patients whose AFP levels failed to respond to treatment with transarterial chemoembolization (TACE) or transarterial radioembolization (TARE) (Riaz et al. 2009). Although the question of whether AFP is useful at predicting the response to sorafenib is controversial (Llovet et al. 2012; Nakazawa et al. 2013), several studies have indicated that AFP response was correlated with time-to-progression (7.9 vs. 2.4 months, $p = 0.004$) and overall survival (13.3 vs. 8.2 months, $p = 0.022$) (Personeni et al. 2012).

AFP-L3 and DCP were also identified as prognostic biomarkers for survival after resection of HCC. Patients who have undergone resection of HCC and who had elevated levels of AFP, AFP-L3, and DCP at the baseline had a worse prognosis than patients who tested positive for just one or two of the markers before surgery (Kiriyama et al. 2011; Nakagawa et al. 2014). Furthermore, several studies have also recently investigated the potential clinical usefulness of DCP in assessing HCC progression. These studies found that (Song et al. 2013c) (i) positivity for serum DCP was significantly related to the presence of vascular invasion, intrahepatic metastasis, tumor size, and TNM stage as well as a high frequency of tumor recurrence, indicating that DCP could serve as an indicator of HCC recurrence after curative therapy; (ii) a high level of DCP is a good predictor of the presence of vascular invasion and could be used to select recipients of liver transplants; and (iii) the use of an inhibitor of DCP in multidrug chemotherapy may induce antiproliferative and antiangiogenic action, indicating that DCP may facilitate the development of new chemotherapeutic strategies for treating HCC.

Among the current guidelines for HCC management worldwide, the guidelines of the NCCN published in 2014 (Benson et al. 2014) recommend high-sectional imaging every 3–6 months for 2 years and then every 6–12 months for posttreatment monitoring. If AFP levels are initially elevated, the guidelines recommend that monitoring be performed every 3 months for 2 years and then every 6–12 months. The Indian National Association for Study of the Liver (INASL) published the first guidelines in India in 2014 (Kumar et al. 2014), and these guidelines make similar recommendations. The guidelines recommend that posttreatment monitoring be performed with dynamic CT or MRI studies every 3 months for the first 2 years and then routine surveillance every 6 months thereafter. The guidelines also note that the serum tumor markers AFP and DCP may help to evaluate the response to treatment or evaluate follow-up when AFP or DCP is elevated at diagnosis and when AFP or DCP decreases after treatment but rises again. The guidelines do note, however, that tumor markers cannot replace imaging modalities. According to the HCC guidelines published in Japan in 2013 (Japan Society of Hepatology 2013), follow-up using the serum biomarkers AFP, AFP-L3, and DCP and imaging should be performed every 3–4 months after treatment. According to NHFPC HCC Guideline published in China in 2011 (National Health and Family Planning Commission 2011), posttreatment monitoring with AFP and imaging should be performed every 3–4 months for 3 years, every 4–6 months for 3–5 years, and then every 6–12 months thereafter if no abnormal findings are detected.

Novel Biomarkers: Potential Applications to Surveillance, Diagnosis, and Prognosis

AFP, AFP-L3, and DCP have been recommended by HCC guidelines in East Asia and elsewhere around the world, but biomarkers such as DKK1, MDK, miRNA, GGT, ICG-R16, GP73, IL-6, SCCA, GPC3, osteopontin, and VEGF are currently being studied to investigate their clinical utility in HCC surveillance, diagnosis, or prognosis.

Of these novel biomarkers, a retrospective, cross-sectional study involving 424 patients with HCC and 407 controls without HCC (213 were healthy, 98 had chronic HBV infection, and 96 had liver cirrhosis) was published in 2012 (Shen et al. 2012); results showed that DKK1 was highly accurate at diagnosing AFP-negative patients with HCC, including patients with early stage HCC. The results also showed that the measurement of DKK1 and AFP together improved the accuracy with which HCC was diagnosed in comparison to any single test alone. These findings add a new piece to the puzzle of diagnosing HCC and they open the door for further investigation of this promising tumor biomarker in independent, prospective studies (Forner and Bruix 2012).

In 2013, a study involving 388 patients with HCC and 545 different controls (Zhu et al. 2013) found that serum MDK had a markedly higher level of sensitivity than AFP (86.9% vs. 51.9%) but a similar level of specificity (83.9% vs. 86.3%). MDK has a significantly higher sensitivity than AFP (80% vs. 40%) at diagnosing very early stage HCC, and its sensitivity could be as high as 89.2% when diagnosing cases of AFP-negative HCC. Serum MDK levels decreased significantly in patients with HCC after curative resection and rose again when the cancer recurred.

The two studies mentioned earlier suggested that the novel serum biomarkers DKK1 and MDK can augment the measurement of AFP when diagnosing HCC, and particularly when diagnosing patients who are negative for AFP and/or who have HCC in an early stage. However, these studies were small in scale and involved few patients. According to the guidelines on phases of evaluating a biomarker for early detection of cancer developed by the National Cancer Institute's Early Detection Research Network (Pepe et al. 2001), more prospective, randomized controlled trials need to be conducted at multiple centers to provide further validation using a larger cohort of serum samples from patients with HCC and hepatitis B and hepatitis C infectious liver disease, nonalcoholic fatty liver disease (NAFLD), and alcohol-induced liver disease (ALD).

Noncoding RNA and microRNA (miRNA) in particular have received considerable attention as novel potential biomarkers over the past few years. Li et al. found that three miRNAs (miR-25, miR-375, and let-7f) could provide a sensitivity of 97.9% and a specificity of 99.1% in diagnosing HCC (Li et al. 2010). Zhou et al. found that a panel of seven microRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801) could provide a high level of diagnostic accuracy for identification of HBV-related HCC (Zhou et al. 2011). Tomimaru et al. found that the combination of miRNA-21 with AFP improved the power of

differentiation between HCC and chronic hepatitis with a sensitivity of 81.0% and a specificity of 80% (Tomimaru et al. 2012).

In addition to their diagnostic potential, miRNAs may help to predict the prognosis for HCC. Tomimaru et al. found that the level of miR-21 expression was high in Asian patients with HCC and that level declined after surgery (Tomimaru et al. 2012). They also found that a high level of miR-21 expression in plasma correlated with a shorter cumulative survival following treatment. Köberle et al. found that in European patients with HCC higher levels of miR-1 and miR-122 expression were associated with longer overall survival compared to lower levels of expression of those miRNAs. They concluded that miR-1 may be a predictive biomarker of HCC independent of liver function. A 31-miRNA signature correlates with the stage of disease (Ura et al. 2009), and a distinct 20-miRNA signature associated with metastasis of HCC has also been identified (Budhu et al. 2008). These findings constitute mounting evidence that miRNA signature profiling can be of use in prognostic stratification. However, the potential for miRNA to serve as a biomarker has not been equally analyzed in all conditions potentially leading to HCC.

Gamma-glutamyl transpeptidase (GGT) has been identified as a prognostic marker in studies of different subgroups of patients published over the past 5 years. Sheen et al. found that patients who had HCC with type B GGT mRNA had worse outcomes, earlier recurrence, and more postrecurrence deaths (Sheen et al. 2003). Several studies of patients with HCC undergoing hepatic resection have revealed a correlation between elevated levels of GGT and worse survival for patients with HBV-related HCC, Child-Pugh A liver function, or multinodular tumors (Ju et al. 2009; Zhao et al. 2013; Zhao et al. 2012). In addition, several studies have also revealed the predictive value of GGT in patients with unresectable HCC who were treated with TACE or chemotherapy (Carr et al. 2010; Guiu et al. 2012; Nishikawa et al. 2013; Zhang et al. 2011). Furthermore, a study published in 2015 examined 384 consecutive cases of curative hepatic resection for single primary HCC to investigate the preoperative predictors of postoperative survival and recurrence (Song et al. 2015). Results showed that GGT $>$ 100 U/L was a preoperative independent risk factor associated with survival, and GGT $>$ 50 U/L and ICG-R15 $>$ 10% were identified as preoperative independent risk factors associated with tumor recurrence. Patients with GGT $>$ 50 U/L and ICG-R15 $>$ 10% had a worse 1-, 3-, and 5-year recurrence-free survival, and this was also true for patients with a tumor $<$ 5 cm in size.

Conclusion

HCC is undoubtedly a great health threat in East Asia since the region has the highest incidence of that cancer, with an age-standardized rate of 31.9 per 100,000 in men. The biomarker AFP has been widely used for routine surveillance and noninvasive

diagnosis of HCC and to evaluate prognosis and monitor recurrence after treatment. In recent years, however, the role of AFP in HCC surveillance and diagnosis has diminished due to advances in imaging modalities. AFP has been excluded from the surveillance and/or diagnostic criteria in HCC guidelines published by the AASLD in 2010, HCC guidelines published by the EASL in 2012, and HCC guidelines published by the NCCN in 2014. Nonetheless, AFP is still regarded as a useful surveillance tool in HCC guidelines in Japan and China and is recommended as an adjunctive tool by HCC guidelines in Japan, China, and South Korea. If the serum AFP level increases steadily over time, the development of HCC should be suspected, and the usual dynamic imaging techniques should be used to make a differential diagnosis.

Advances in technology and an increased understanding of the pathology of HCC have led to the discovery of novel biomarkers. Data have indicated that the combined testing of AFP, AFP-L3, or DCP could help to increase the sensitivity of diagnosis of HCC, but this approach is currently used in only a few countries, such as Japan. In recent years, numerous studies have investigated the clinical usefulness of some novel biomarkers in the early diagnosis of HCC, including DKK1, MDK, and miRNA. Moreover, the prognostic significance of some biomarkers, such as miRNA, GGT, and ICG-R15, has also been evaluated. However, further studies are needed to better characterize the accuracy and potential role of these approaches in clinical practice. The prevailing hope is that novel biomarkers can support clinicians in their daily practice and improve care for patients with HCC.

Summary Points

- This chapter has focused on the clinical utility of biomarkers for hepatocellular carcinoma (HCC) in East Asia, including Japan, China, and South Korea.
- Alpha-fetoprotein (AFP) testing and abdominal ultrasound (US) every 6 months are recommended for routine surveillance of HCC in high-risk patients, and AFP has also been used as a diagnostic test for HCC and to evaluate prognosis and monitor recurrence following treatment.
- However, controversy regarding the clinical utility of AFP has arisen in the West and the East in recent years. This controversy is also evident in HCC guidelines in countries in East Asia
- Data have indicated that the combined testing of AFP, the lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), or des-γ-carboxyprothrombin (DCP) could help to increase the sensitivity of diagnosis of HCC, but this approach is currently used in only a few countries, such as Japan.
- Numerous studies have investigated the clinical usefulness of some novel biomarkers in early diagnosis of HCC, including Dickkopf-1 (DKK1), midkine (MDK), and microRNA (miRNA).
- The prognostic significance of some biomarkers, such as miRNA, gamma-glutamyl transferase (GGT), and indocyanine green retention 15 min after administration (ICG-R15), has also been evaluated.

- However, further studies are needed to better characterize the accuracy and potential role of these approaches in clinical practice.

References

Benson 3rd AB, D'angelica MI, Abrams TA, Are C, Bloomston PM, Chang DT, Clary BM, Covey AM, Ensminger WD, Iyer R, Kelley RK, Linehan D, Malafa MP, Meranze SG, Park JO, Pawlik T, Posey JA, Scaife C, Schefter T, Sigurdson ER, Tian GG, Vauthey JN, Venook AP, Yen Y, Zhu AX, Hoffmann KG, Mcmillian NR, Sundar H. Hepatobiliary cancers, version 2.2014. J Natl Compr Cancer Netw. 2014;12:1152–82.

Bertino G, Ardiri A, Malaguarnera M, Malaguarnera G, Bertino N, Calvagno GS. Hepatocellualar carcinoma serum markers. Semin Oncol. 2012;39:410–33.

Bolondi L. Screening for hepatocellular carcinoma in cirrhosis. J Hepatol. 2003;39:1076–84.

Bruix J, Sherman M, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. Hepatology. 2011;53:1020–2.

Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, Zanetti KA, Ye QH, Qin LX, Croce CM, Tang ZY, Wang XW. Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology. 2008;47:897–907.

Carr BI, Pancoska P, Branch RA. Low alpha-fetoprotein hepatocellular carcinoma. J Gastroenterol Hepatol. 2010;25:1543–9.

Chinese Anti-Cancer Association Society of Liver Cancer, C. S. O. C. O., Chinese Society of Hepatology, Liver Cancer Study Group. The expert consensus on the treatment standards for hepatocellular carcinoma. Dig Dis Endosc. 2009;3:40–51.

Chung H, Ueda T, Kudo M. Changing trends in hepatitis C infection over the past 50 years in Japan. Intervirology. 2010;53:39–43.

Clinical practice guidelines for hepatocellular carcinoma – The Japan Society of Hepatology 2009 update. Hepatol Res. 2010;40(Suppl 1):2–144.

Cui R, Wang B, Ding H, Shen H, Li Y, Chen X. Usefulness of determining a protein induced by vitamin K absence in detection of hepatocellular carcinoma. Chin Med J (Engl). 2002;115:42–5.

Cui R, He J, Zhang F, Wang B, Ding H, Shen H, Li Y, Chen X. Diagnostic value of protein induced by vitamin K absence (PIVKAII) and hepatoma-specific band of serum gamma-glutamyl transferase (GGTII) as hepatocellular carcinoma markers complementary to alpha-fetoprotein. Br J Cancer. 2003;88:1878–82.

European Association for the Study of the Liver, European Organisation for Research & Treatment of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol. 2012;56:908–43.

Forner A, Bruix J. Biomarkers for early diagnosis of hepatocellular carcinoma. Lancet Oncol. 2012;13:750–1.

Gao JJ, Song PP, Tamura S, Hasegawa K, Sugawara Y, Kokudo N, Uchida K, Orii R, Qi FH, Dong JH, Tang W. Standardization of perioperative management on hepato-biliary-pancreatic surgery. Drug Discov Ther. 2012;6:108–11.

Guiu B, Deschamps F, Boulin M, Boige V, Malka D, Ducreux M, Hillon P, De Baere T. Serum gamma-glutamyl-transferase independently predicts outcome after transarterial chemoembolization of hepatocellular carcinoma: external validation. Cardiovasc Intervent Radiol. 2012;35:1102–8.

Ikai I, Arii S, Okazaki M, Okita K, Omata M, Kojiro M, Takayasu K, Nakanuma Y, Makuuchi M, Matsuyama Y, Monden M, Kudo M. Report of the 17th Nationwide Follow-up Survey of Primary Liver Cancer in Japan. Hepatol Res. 2007;37:676–91.

Ioannou GN, Splan MF, Weiss NS, Mcdonald GB, Beretta L, Lee SP. Incidence and predictors of hepatocellular carcinoma in patients with cirrhosis. Clin Gastroenterol Hepatol. 2007;5:938–45, 945 e1–4.

Japan Society of Hepatology. Clinical practice guidelines for hepatocellular carcinoma (2013 version). Tokyo: Kanehara; 2013 (in Japanese).

Ju MJ, Qiu SJ, Fan J, Zhou J, Gao Q, Cai MY, Li YW, Tang ZY. Preoperative serum gamma-glutamyl transferase to alanine aminotransferase ratio is a convenient prognostic marker for Child-Pugh A hepatocellular carcinoma after operation. J Gastroenterol. 2009;44:635–42.

Kim MJ, Bae KW, Seo PJ, Jeong IK, Kim JH, Lee BH, Bang KT, Kim DW, Song IH. Optimal cut-off value of PIVKA-II for diagnosis of hepatocellular carcinoma–using ROC curve. Korean J Hepatol. 2006;12:404–11.

Kiriyama S, Uchiyama K, Ueno M, Ozawa S, Hayami S, Tani M, Yamaue H. Triple positive tumor markers for hepatocellular carcinoma are useful predictors of poor survival. Ann Surg. 2011;254:984–91.

Korean Liver Cancer Study Group & National Cancer Center, Korea. Practice guidelines for management of hepatocellular carcinoma 2009. Korean J Hepatol. 2009;15:391–423.

Korean Liver Cancer Study Group & National Cancer Center, Korea. 2014 KLCSG-NCC Korea practice guideline for the management of hepatocellular carcinoma. Gut Liver. 2015a;9:267–317.

Korean Liver Cancer Study Group & National Cancer Center, Korea. 2014 Korean Liver Cancer Study Group-National Cancer Center Korea practice guideline for the management of hepatocellular carcinoma. Korean J Radiol. 2015b;16:465–522.

Kudo M. Real practice of hepatocellular carcinoma in Japan: conclusions of the Japan Society of Hepatology 2009 Kobe Congress. Oncology. 2010;78 Suppl 1:180–8.

Kumar A, Acharya SK, Singh SP, Saraswat VA, Arora A, Duseja A, Goenka MK, Jain D, Kar P, Kumar M, Kumaran V, Mohandas KM, Panda D, Paul SB, Ramachandran J, Ramesh H, Rao PN, Shah SR, Sharma H, Thandassery RB. The Indian National Association for Study of the Liver (INASL) consensus on prevention, diagnosis and management of hepatocellular carcinoma in India: the Puri recommendations. J Clin Exp Hepatol. 2014;4:S3–26.

Kwak HW, Park JW, Nam BH, Yu A, Woo SM, Kim TH, Kim SH, Koh YH, Kim HB, Park SJ, Lee WJ, Hong EK, Kim CM. Clinical outcomes of a cohort series of patients with hepatocellular carcinoma in a hepatitis B virus-endemic area. J Gastroenterol Hepatol. 2014;29:820–9.

Li LM, Hu ZB, Zhou ZX, Chen X, Liu FY, Zhang JF, Shen HB, Zhang CY, Zen K. Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. Cancer Res. 2010;70:9798–807.

Llovet JM, Bruix J. Novel advancements in the management of hepatocellular carcinoma in 2008. J Hepatol. 2008;48 Suppl 1:S20–37.

Llovet JM, Pena CE, Lathia CD, Shan M, Meinhardt G, Bruix J, SHARP Investigators Study Group. Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. Clin Cancer Res. 2012;18:2290–300.

Mailey B, Artinyan A, Khalili J, Denitz J, Sanchez-Luege N, Sun CL, Bhatia S, Nissen N, Colquhoun SD, Kim J. Evaluation of absolute serum alpha-fetoprotein levels in liver transplant for hepatocellular cancer. Arch Surg. 2011;146:26–33.

Makuuchi M, Kokudo N. Clinical practice guidelines for hepatocellular carcinoma: the first evidence based guidelines from Japan. World J Gastroenterol. 2006;12:828–9.

Mitsuhashi N, Kobayashi S, Doki T, Kimura F, Shimizu H, Yoshidome H, Ohtsuka M, Kato A, Yoshitomi H, Nozawa S, Furukawa K, Takeuchi D, Suda K, Miura S, Miyazaki M. Clinical significance of alpha-fetoprotein: involvement in proliferation, angiogenesis, and apoptosis of hepatocellular carcinoma. J Gastroenterol Hepatol. 2008;23:e189–97.

Nakagawa S, Beppu T, Okabe H, Sakamoto K, Kuroki H, Mima K, Nitta H, Imai K, Hayashi H, Sakamoto Y, Hashimoto D, Chikamoto A, Ishiko T, Watanabe M, Baba H. Triple positive tumor markers predict recurrence and survival in early stage hepatocellular carcinoma. Hepatol Res. 2014;44:964–74.

Nakazawa T, Hidaka H, Takada J, Okuwaki Y, Tanaka Y, Watanabe M, Shibuya A, Minamino T, Kokubu S, Koizumi W. Early increase in alpha-fetoprotein for predicting unfavorable clinical

outcomes in patients with advanced hepatocellular carcinoma treated with sorafenib. Eur J Gastroenterol Hepatol. 2013;25:683–9.
Korean Liver Cancer Study Group & National Cancer Center, Korea. 2014 KLCSG-NCC Korea practice guideline for the management of hepatocellular carcinoma. Gut Liver.2015a; 9:267–317.
National Health and Family Planning Commission. The guideline on diagnosis and treatment for primary liver cancer http://www.moh.gov.cn/mohyzs/s3586/201110/53153.shtml 2011 (in Chinese)
Nguyen MH, Garcia RT, Simpson PW, Wright TL, Keeffe EB. Racial differences in effectiveness of alpha-fetoprotein for diagnosis of hepatocellular carcinoma in hepatitis C virus cirrhosis. Hepatology. 2002;36:410–7.
Nishikawa H, Nishijima N, Arimoto A, Inuzuka T, Kita R, Kimura T, Osaki Y. Prognostic factors in patients with hepatitis B virus-related hepatocellular carcinoma undergoing nucleoside analog antiviral therapy. Oncol Lett. 2013;6:1213–8.
Omata M, Lesmana LA, Tateishi R, Chen PJ, Lin SM, Yoshida H, Kudo M, Lee JM, Choi BI, Poon RT, Shiina S, Cheng AL, Jia JD, Obi S, Han KH, Jafri W, Chow P, Lim SG, Chawla YK, Budihusodo U, Gani RA, Lesmana CR, Putranto TA, Liaw YF, Sarin SK. Asian Pacific Association for the Study of the Liver consensus recommendations on hepatocellular carcinoma. Hepatol Int. 2010;4:439–74.
Park JW, Korean Liver Cancer Study Group & National Cancer Center. Practice guideline for diagnosis and treatment of hepatocellular carcinoma. Korean J Hepatol. 2004;10:88–98.
Pepe MS, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, Winget M, Yasui Y. Phases of biomarker development for early detection of cancer. J Natl Cancer Inst. 2001;93:1054–61.
Personeni N, Bozzarelli S, Pressiani T, Rimassa L, Tronconi MC, Sclafani F, Carnaghi C, Pedicini V, Giordano L, Santoro A. Usefulness of alpha-fetoprotein response in patients treated with sorafenib for advanced hepatocellular carcinoma. J Hepatol. 2012;57:101–7.
Riaz A, Ryu RK, Kulik LM, Mulcahy MF, Lewandowski RJ, Minocha J, Ibrahim SM, Sato KT, Baker T, Miller FH, Newman S, Omary R, Abecassis M, Benson 3rd AB, Salem R. Alpha-fetoprotein response after locoregional therapy for hepatocellular carcinoma: oncologic marker of radiologic response, progression, and survival. J Clin Oncol. 2009;27:5734–42.
Rich N, Singal AG. Hepatocellular carcinoma tumour markers: current role and expectations. Best Pract Res Clin Gastroenterol. 2014;28:843–53.
Shaheen MA, Idrees M. Evidence-based consensus on the diagnosis, prevention and management of hepatitis C virus disease. World J Hepatol. 2015;7:616–27.
Sheen IS, Jeng KS, Tsai YC. Is the expression of gamma-glutamyl transpeptidase messenger RNA an indicator of biological behavior in recurrent hepatocellular carcinoma? World J Gastroenterol. 2003;9:468–73.
Shen Q, Fan J, Yang XR, Tan Y, Zhao W, Xu Y, Wang N, Niu Y, Wu Z, Zhou J, Qiu SJ, Shi YH, Yu B, Tang N, Chu W, Wang M, Wu J, Zhang Z, Yang S, Gu J, Wang H, Qin W. Serum DKK1 as a protein biomarker for the diagnosis of hepatocellular carcinoma: a large-scale, multicentre study. Lancet Oncol. 2012;13:817–26.
Singal A, Volk ML, Waljee A, Salgia R, Higgins P, Rogers MA, Marrero JA. Meta-analysis: surveillance with ultrasound for early-stage hepatocellular carcinoma in patients with cirrhosis. Aliment Pharmacol Ther. 2009;30:37–47.
Song P. Standardizing management of hepatocellular carcinoma in China: devising evidence-based clinical practice guidelines. Biosci Trends. 2013;7:250–2.
Song P, Tobe RG, Inagaki Y, Kokudo N, Hasegawa K, Sugawara Y, Tang W. The management of hepatocellular carcinoma around the world: a comparison of guidelines from 2001 to 2011. Liver Int. 2012;32:1053–63.
Song P, Feng X, Zhang K, Song T, Ma K, Kokudo N, Dong J, Tang W. Perspectives on using des-gamma-carboxyprothrombin (DCP) as a serum biomarker: facilitating early detection of hepatocellular carcinoma in China. Hepatobiliary Surg Nutr. 2013a;2:227–31.

Song P, Feng X, Zhang K, Song T, Ma K, Kokudo N, Dong J, Yao L, Tang W. Screening for and surveillance of high-risk patients with HBV-related chronic liver disease: promoting the early detection of hepatocellular carcinoma in China. Biosci Trends. 2013b;7:1–6.

Song P, Gao J, Inagaki Y, Kokudo N, Hasegawa K, Sugawara Y, Tang W. Biomarkers: evaluation of screening for and early diagnosis of hepatocellular carcinoma in Japan and china. Liver Cancer. 2013c;2:31–9.

Song P, Feng X, Inagaki Y, Song T, Zhang K, Wang Z, Zheng S, Ma K, Li Q, Kong D, Wu Q, Zhang T, Zhao X, Hasegawa K, Sugawara Y, Kokudo N, Tang W, Japan-China Joint Team for Medical Research & Cooperation on HCC. Clinical utility of simultaneous measurement of alpha-fetoprotein and des-gamma-carboxy prothrombin for diagnosis of patients with hepatocellular carcinoma in China: A multi-center case-controlled study of 1,153 subjects. Biosci Trends. 2014a;8:266–73.

Song P, Tang W, Hasegawa K, Kokudo N. Systematic evidence-based clinical practice guidelines are ushering in a new stage of standardized management of hepatocellular carcinoma in Japan. Drug Discov Ther. 2014b;8:64–70.

Song P, Inagaki Y, Wang Z, Hasegawa K, Sakamoto Y, Arita J, Tang W, Kokudo N. High levels of gamma-glutamyl transferase and indocyanine green retention rate at 15 min as preoperative predictors of tumor recurrence in patients with hepatocellular carcinoma. Medicine (Baltimore). 2015;94:e810.

Stravitz RT, Heuman DM, Chand N, Sterling RK, Shiffman ML, Luketic VA, Sanyal AJ, Habib A, Mihas AA, Giles HC, Maluf DG, Cotterell AH, Posner MP, Fisher RA. Surveillance for hepatocellular carcinoma in patients with cirrhosis improves outcome. Am J Med. 2008;121:119–26.

Tanaka M, Katayama F, Kato H, Tanaka H, Wang J, Qiao YL, Inoue M. Hepatitis B and C virus infection and hepatocellular carcinoma in China: a review of epidemiology and control measures. J Epidemiol. 2011;21:401–16.

Tandon P, Garcia-Tsao G. Prognostic indicators in hepatocellular carcinoma: a systematic review of 72 studies. Liver Int. 2009;29:502–10.

Tateishi R, Yoshida H, Matsuyama Y, Mine N, Kondo Y, Omata M. Diagnostic accuracy of tumor markers for hepatocellular carcinoma: a systematic review. Hepatol Int. 2008;2:17–30.

Tomimaru Y, Eguchi H, Nagano H, Wada H, Kobayashi S, Marubashi S, Tanemura M, Tomokuni A, Takemasa I, Umeshita K, Kanto T, Doki Y, Mori M. Circulating microRNA-21 as a novel biomarker for hepatocellular carcinoma. J Hepatol. 2012;56:167–75.

Ura S, Honda M, Yamashita T, Ueda T, Takatori H, Nishino R, Sunakozaka H, Sakai Y, Horimoto K, Kaneko S. Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma. Hepatology. 2009;49:1098–112.

WHO. Globocan 2012: estimated cancer incidence, mortality and prevalence worldwide in 2012. http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx (2012). Accessed 2 Mar 2015

Yang JD, Harmsen WS, Slettedahl SW, Chaiteerakij R, Enders FT, Therneau TM, Orsini L, Kim WR, Roberts LR. Factors that affect risk for hepatocellular carcinoma and effects of surveillance. Clin Gastroenterol Hepatol. 2011;9:617–23 e1.

Yoon YJ, Han KH, Kim C, Chon CY, Moon YM, Han CH, Choi HJ, Kim YS, Han JY, Kim HS. Clinical efficacy of serum PIVKA-II in the diagnosis and follow up after treatment of hepatocellular carcinoma. Taehan Kan Hakhoe Chi. 2002;8:465–71.

Yuen MF, Hou JL, Chutaputti A, Asia Pacific Working Party on Prevention of Hepatocellular Carcinoma. Hepatocellular carcinoma in the Asia pacific region. J Gastroenterol Hepatol. 2009;24:346–53.

Zhang BH, Yang BH, Tang ZY. Randomized controlled trial of screening for hepatocellular carcinoma. J Cancer Res Clin Oncol. 2004;130:417–22.

Zhang JB, Chen Y, Zhang B, Xie X, Zhang L, Ge N, Ren Z, Ye SL. Prognostic significance of serum gamma-glutamyl transferase in patients with intermediate hepatocellular carcinoma treated with transcatheter arterial chemoembolization. Eur J Gastroenterol Hepatol. 2011;23:787–93.

Zhang C, Zhong Y, Guo L. Strategies to prevent hepatitis B virus infection in China: immunization, screening, and standard medical practices. Biosci Trends. 2013;7:7–12.
Zhao WC, Zhang HB, Yang N, Fu Y, Qian W, Chen BD, Fan LF, Yang GS. Preoperative predictors of short-term survival after hepatectomy for multinodular hepatocellular carcinoma. World J Gastroenterol. 2012;18:3272–81.
Zhao WC, Fan LF, Yang N, Zhang HB, Chen BD, Yang GS. Preoperative predictors of microvascular invasion in multinodular hepatocellular carcinoma. Eur J Surg Oncol. 2013;39:858–64.
Zhou J, Yu L, Gao X, Hu J, Wang J, Dai Z, Wang JF, Zhang Z, Lu S, Huang X, Wang Z, Qiu S, Wang X, Yang G, Sun H, Tang Z, Wu Y, Zhu H, Fan J. Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. J Clin Oncol. 2011;29:4781–8.
Zhu WW, Guo JJ, Guo L, Jia HL, Zhu M, Zhang JB, Loffredo CA, Forgues M, Huang H, Xing XJ, Ren N, Dong QZ, Zhou HJ, Ren ZG, Zhao NQ, Wang XW, Tang ZY, Qin LX, Ye QH. Evaluation of midkine as a diagnostic serum biomarker in hepatocellular carcinoma. Clin Cancer Res. 2013;19:3944–54.

Monocyte Chemotactic Protein-1 (Cytokine, Receptors, and Gene Polymorphisms) in Hepatitis

43

Alicja E. Grzegorzewska and Adrianna Mostowska

Contents

Abstract

In this review, we have described monocyte chemotactic protein-1 (MCP-1), its receptors, and polymorphisms of genes encoding MCP-1 and its receptors in relation to hepatitis of different etiologies. The *MCP1* -2518 G allele is associated with upregulation of *MCP-1* transcript, and subjects bearing the G allele produce

A.E. Grzegorzewska (✉)
Department of Nephrology, Transplantology and Internal Diseases, Poznan University of Medical Sciences, Poznań, Poland
e-mail: alicja_grzegorzewska@yahoo.com

A. Mostowska (✉)
Department of Biochemistry and Molecular Biology, Poznan University of Medical Sciences, Poznań, Poland
e-mail: amostowska@wp.pl

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_8

more MCP-1 that is mainly involved in leukocyte recruitment and trafficking. The cell receptors that bind MCP-1 are CCR2 and CCR5. Polymorphisms of genes encoding receptor proteins may potentially alter MCP-1 affinity to receptors and decrease MCP-1 relevance.

Available results are suggestive that the G allele in *MCP1* -2518 A/G is a risk allele for worse hepatitis C course and for higher severity of alcoholic hepatitis. *CCR5* polymorphisms or mutations influence resolution of HCV/HBV infections and predict response to treatment of hepatitis C with interferon-alpha. Expression of MCP-1 in hepatic tissue specimens shows correlation with severity of liver disease and hepatic fibrosis. Direct correlations between serum MCP-1 concentration, hepatic expression of *MCP1*, and *MCP1* -2518 G allele are usually not seen in hepatitis. Tested *CCR2* polymorphisms were not predictive either for resolution of viral infections or hepatitis severity.

Larger randomized studies could be confirmative or not for establishment of many interesting results signalizing by MCP-1-associated data performed usually in groups showing too small sample power for definitive conclusions.

Keywords

Hepatitis • Hepatitis B virus • Hepatitis C virus • Monocyte chemotactic protein-1 • Polymorphisms • Receptors • Treatment responsiveness

List of Abbreviations

ALT	Alanine aminotransferase
anti-HBs	Antibodies to surface antigen of hepatitis B virus
anti-HCV	Antibodies to hepatitis C virus
AST	Aspartate aminotransferase
DARC	Duffy antigen receptor for chemokines
DNA	Deoxyribonucleic acid
GWAS	Genome-wide association study
HBsAg	Surface antigen of hepatitis B virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HD	Hemodialysis
HLA	Human leukocyte antigen
HWE	Hardy–Weinberg equilibrium
IFN	Interferon
IL	Interleukin
LD	Linkage disequilibrium
MAF	Minor allele frequency
MCP1	Monocyte chemotactic protein-1 gene
MCP-1	Monocyte chemotactic protein-1
MIP	Macrophage inflammatory protein

mRNA	Messenger ribonucleic acid
NK	Natural killer
OMIM	Online Mendelian Inheritance in Man
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
Th	T helper
TNF	Tumor necrosis factor

Key Facts of *MCP1* -2518 A/G

1. The *MCP1* -2518 A/G (rs1024611) polymorphism is located in the functional promoter region of *MCP1*.
2. Monocytes from individuals carrying the G allele at -2518 produce more monocyte chemotactic protein-1 (MCP-1) in response to inflammatory stimuli.
3. Binding of transcription factors MyoD and AP-4 was indicated only in the presence of nucleotide G, not in the presence of nucleotide A at position -2528 relative to the transcription site.
4. Distribution of the risk G allele varies worldwide: from 23% in Africans to 55% Asians.
5. Components of the IL-6 receptor complex are essential for MCP-1 expression: STAT3, CEBP-alpha, and PU.1 were indicated as major players in IL-6-mediated MCP-1 induction.
6. MCP-1 is a pro-inflammatory and pro-fibrotic chemokine.
7. MCP-1 is a ligand for CC chemokine receptor type 2 (CCR2) and CC chemokine receptor type 5 (CCR5).
8. The Duffy antigen receptor for chemokines contributes to posttranslational regulation of MCP-1 concentration in serum and tissues.
9. Serum levels of MCP-1 increase with deterioration of renal function.

Definitions of Words and Terms

Chemokine	*Cytokine* that has the ability to attract cells to sites of infection/inflammation
Cytokine	A small protein that is a *ligand* for specific *receptor*(*s*) and affects the behavior of other cells by cell signaling
Ligand	A small molecule that has an affinity to a specific receptor protein, alters its chemical conformation, and determines its functional state
Mutation	A permanent change of the nucleotide sequence of the genome that may alter the product of a gene

Polymorphism	The existence of many forms of DNA sequences at a locus within the population
Receptor	A protein molecule that binds ligand(s) of a particular structure which activates or inhibits the receptor's associated biochemical pathway

Introduction

Monocyte Chemotactic Protein-1 Gene (*MCP1*)

The gene encoding monocyte chemotactic protein-1 (MCP-1) is referred to as *MCP1*, *CCL2*, or *SCYA2* (OMIM +158105). In humans, *MCP1* is located on chromosome 17 (Fig. 1). Linkage disequilibrium (LD) plot of HapMap single nucleotide polymorphisms (SNPs) within the *MCP1* locus is shown in Fig. 2. Two polymorphisms have been identified in the distal regulatory region of *MCP1*. The polymorphism at -2076 does not appear to affect *MCP1* transcription. The *MCP1* -2518 A/G polymorphism is located in the functional promoter region of *MCP1*. Its identification number of a reference SNP is rs1024611. The *MCP1* product is chemokine MCP-1. In vitro, monocytes from individuals carrying the G allele at -2518 produced more MCP-1 in response to inflammatory stimuli [interleukin (IL)-1 beta] than cells from subjects possessing AA genotype (Rovin et al. 1999). Mühlbauer et al. (2003) identified potential binding sites of the transcription factors MyoD and AP-4. Binding of both transcription factors was indicated only in the presence of nucleotide G, not in the presence of nucleotide A at position -2528 relative to the transcription site. The *MCP1*-2518 G allele was associated with upregulation of both *MCP1* transcript and protein levels also in later studies (Fenoglio et al. 2004; Flores-Villanueva et al. 2005; Buraczyńska et al. 2008; Xu et al. 2009).

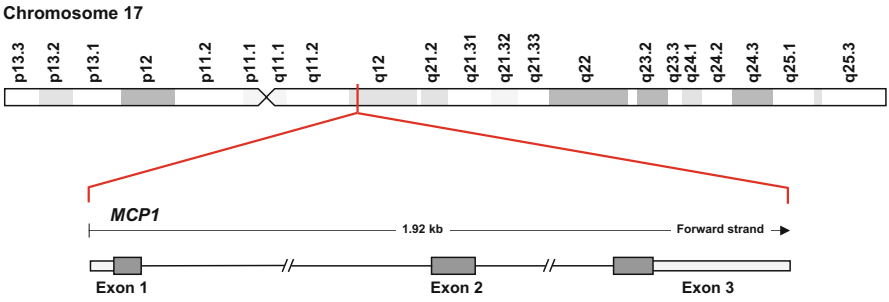

Fig. 1 Chromosomal localization and the structure of *MCP1* encoding the monocyte chemotactic protein-1

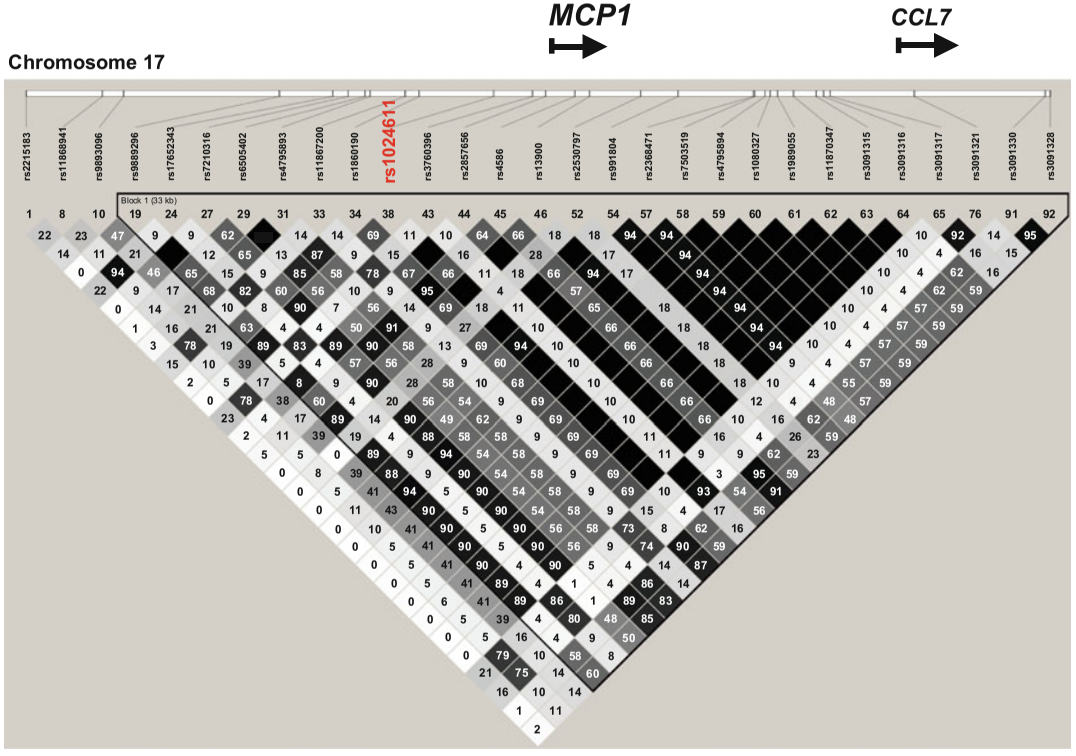

Fig. 2 Linkage disequilibrium (LD) plot of HapMap SNPs within the *MCP1* locus. The plot was generated using genotype data from HapMap CEU samples and the Haploview 4.0 software (Broad Institute, Cambridge, MA). Numbers denote r^2 values expressed as a percentage of maximal value (1.0). Squares without numbers correspond to $r^2 = 1.0$. A black-to-white gradient shows highest (1.0) to lowest (0.0) r^2. The functional polymorphism rs1024611 (-2518A/G) located in the promoter region of the *MCP1* is marked in red. The *MCP1* encodes the monocyte chemotactic protein-1. The *CCL7* gene encodes the monocyte chemotactic protein 3

Table 1 Distribution of *MCP1* -2518 A>G genotypes in the healthy population of different countries

Studies in controls	Country	*MCP1* -2518 A>G rs1024611		
		AA	AG	GG
Ben-Selma et al. (2011)	Tunisia	93 (0.62)	49 (0.33)	8 (0.05)
Buraczyńska et al. (2008)	Poland (Lubelskie)	209 (0.64)	101 (0.31)	15 (0.05)
Flex et al. (2004)	Italy	124 (0.56)	87 (0.39)	12 (0.05)
Flores-Villanueva et al. (2005)	Korea	66 (0.41)	74 (0.46)	22 (0.13)
Karadeniz et al. (2010)	Turkey	49 (0.47)	44 (0.42)	12 (0.11)
Ksiaa Cheikh Rouhou et al. (2011)	Tunisia	91 (0.53)	67 (0.40)	12 (0.17)
Mostowska et al. (2012)	Poland (Wielkopolska)	225 (0.51)	177 (0.41)	35 (0.08)
Mühlbauer et al. (2003)	Germany	71 (0.51)	59 (0.43)	9 (0.06)
Salama et al. (2014)	Egypt	11 (0.55)	8 (0.40)	1 (0.05)
Szalai et al. (2001a)	Hungary	186 (0.58)	115 (0.36)	19 (0.06)
Xu et al. (2009)	China	41 (0.41)	45 (0.45)	14 (0.14)
Yang et al. (2004)	United Kingdom	36 (0.35)	60 (0.58)	8 (0.07)

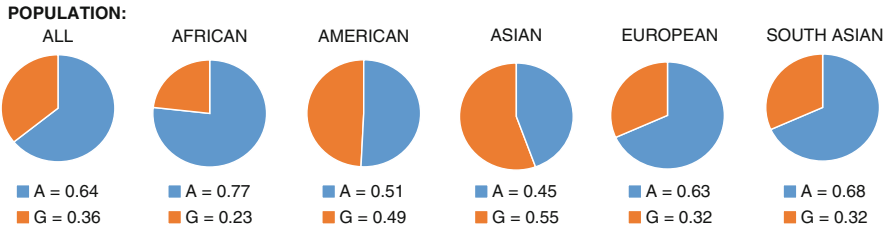

Fig. 3 The allele frequency of the *MCP1* rs1024611 functional polymorphism in different populations (According to the 1000 Genomes database)

Distribution of *MCP1* -2518 A/G genotypes in the healthy subjects of different countries or populations is shown in Table 1 and in Fig. 3, respectively. It is noteworthy that the risk G allele distribution varies worldwide: from 23% in Africans to 55% Asians.

Monocyte Chemotactic Protein-1 (MCP-1)

MCP-1 is also known as chemokine (CC motif) ligand 2 (CCL2), small inducible cytokine A2, monocyte chemoattractant protein-1, monocyte chemotactic and activating factor, and monocyte secretory protein. This chemokine is produced as a protein precursor containing signal peptide of 23 amino acids and a mature peptide of 76 amino acids. It is a monomeric polypeptide, with a molecular weight of approximately 11 kDa. MCP-1 belongs to the chemokine family that includes small polypeptides with a significant role in leukocyte recruitment and trafficking. Expression of MCP-1 can be induced in many cell types, including inflammatory

cells, hepatocytes, liver-resident macrophages (Kupffer cells), and stellate cells (Marra et al. 1999; Simpson et al. 2003). Hepatic stellate cells have been shown to regulate leukocyte trafficking by secreting MCP-1 (Czaja et al. 1994). MCP-1 acts as a chemotactic factor for monocytes/macrophages, activated lymphocytes, natural killer cells, stellate cells, and neutrophils during infections (Rollins et al. 1991; Rollins 1996; Lu et al. 1998; Marra et al. 1999; Kolattukudy and Niu 2012). MCP-1 is also expressed in bone cells and is under the control of nuclear factor kappaB (Kim et al. 2005).

Chemokine serum and tissue concentrations are determined by transcriptional activity and by posttranslational regulation.

Components of the IL-6 receptor complex are essential for MCP-1 expression (Sarma et al. 2014). A 4.7-fold increase in the MCP-1 reporter activity was observed in IL-6-treated cells compared to the untreated cells, whereas knockdown of the IL-6 cell surface receptor gene resulted in a twofold decrease in IL-6-mediated MCP-1 activation and protein expression compared to controls. Knockdown of *JAK1* resulted in a 1.8-fold decrease of IL-6-mediated MCP-1 reporter activity and protein expression. STAT3, CEBP-alpha, and PU.1 were indicated as major players in IL-6-mediated MCP-1 induction (Sarma et al. 2014).

Chemokine scavenger or decoy receptors contribute to posttranslational regulation. These receptors include D6, CCX-CKR, and the Duffy antigen receptor for chemokines (DARC) (Mantovani et al. 2006). A nonsynonymous SNP in the DARC gene (*DARC* rs12075, p.Asp42Gly) was shown to be a major determinant of MCP-1 serum concentration in a genome-wide association study (GWAS) and family-based linkage analysis. This SNP in *DARC* accounted for 20% of the variability in MCP-1 serum concentrations and showed a high frequency of the minor allele (MAF) (45.6%) among Caucasian populations (Schnabel et al. 2009).

Serum levels of MCP-1 increase with deterioration of renal function. In majority of hemodialysis (HD) individuals, they are higher than in healthy individuals (Papayianni et al. 2002; Buraczyńska et al. 2008; Uchida et al. 2012). MCP-1 levels were higher in HD subjects with AG+GG genotypes of *MCP1* rs1024611 than those in HD patients with the AA variant (Buraczyńska et al. 2008).

MCP-1 Receptors and Their Genes

The cell surface receptors that bind MCP-1 are CC chemokine receptor type 2 (CCR2, OMIM *601267) and CC chemokine receptor type 5 (CCR5, OMIM *601373).

CCR2 is expressed on monocytes, T lymphocytes, and basophils (Sallusto et al. 1998; Simpson et al. 2003). CCR2 binds MCP-1, MCP-3, MCP-5, CCL8, and CCL16 (Franci et al. 1995; Sarafi et al. 1997; Nomiyama et al. 2001).

The CCR2 gene (*CCR2*) is located on the chromosome 3p21, closely to another chemokine receptor gene – *CCR5*. The G to A transition at nucleotide position +190 leads to an exchange of neutrally charged amino acids (valine to isoleucine) in the first transmembrane domain of CCR2. p.Val64Ile represents a conservative change.

This mutation in *CCR2* is designed *CCR2* p.Val64Ile and also referred to *CCR2* G190A (rs1799864, MAF = 0.108, HapMap CEU cohort). The receptor containing the variance has been shown to be expressed efficiently on the cell surface. It transduces signals in response to MCP-1 binding and protein expression is not altered (Lee et al. 1998). In the light of these data, affinity of CCR2 for its ligand (MCP-1) should not be changed. Another variant in *CCR2*, tested in patients showing hepatitis C virus (HCV) infection, was T780C (p.Asn260Asn) (Mascheretti et al. 2004).

CCR5 is a CC chemokine receptor expressed by T helper (Th) cells, mainly Th1 cells, and also memory and activated T cells, CD8 lymphocytes, granulocytes, macrophages, and immature dendritic cells. It plays an important role in T-cell differentiation, and it facilitated antigen-primed T-cell migration and activation (Ansel et al. 1999). CCR5 expression depends on the activation state of T cells and is upregulated by IL-2 (Sallusto et al. 1998). CCR5 is a receptor for MCP-1, macrophage inflammatory protein-1a (MIP-1a), MIP-1b, RANTES, CCL3, CCL4, CCL5, CCL11, CCL13, CCL14, and CCL16 (Hellier et al. 2003; Nomiyama et al. 2001). CCR5 is partly responsible for the recruitment of T cells to the portal region in hepatitis. Impaired macrophage and T-cell function and reduced clearance of intracellular pathogens were described in CCR5 knockout mice (Zhou et al. 1998). CCR5 knockout mice displayed a Th2 immune response (Zhou et al. 1998; Andres et al. 2000). CCR5 deficiency in mice causes a more robust T-cell response to infectious agents like *Mycobacterium tuberculosis* (Algood and Flynn 2004).

The gene encoding the CCR5 protein (*CCR5*) is located on chromosome 3p21 and consists of a single coding exon. The most frequently tested *CCR5* variants are *CCR5*-Δ32 (rs333) and *CCR5*-59029 A/G (rs1799988). The 32 base pair (bp) deletion in exon 4 of *CCR5* leads to premature termination of the protein. The resultant CCR5 mutant protein is likely to be functionally inert as it not only lacks the last three of seven putative transmembrane regions but also the domains involved in G protein coupling and signal transduction (Samson et al. 1996). A truncated protein fails to reach the cell surface and is not detectable at the surface of cells that would normally express it and therefore cannot act as a receptor (Liu et al. 1996). Individuals homozygous for this mutation (1% of Caucasians) cannot express CCR5 on the cell surface, whereas the heterozygous state (10–15% of Caucasians) results in decreased expression of the functional CCR5 (Liu et al. 1996; Wu et al. 1997; Thio et al. 2007). *CCR5*-Δ32 is associated with reduced migration of circulating lymphocytes (Liu et al. 1996). The *CCR5* promoter containing the 59029 G allele has 45% lower activity compared to the promoter containing the 59029 A allele (McDermott et al. 1998). Strong linkage disequilibrium between the *CCR5* -59029 and the *CCR5* -59353 polymorphic variants was identified (Chang et al. 2005).

MCP-1 and CCR2 Functional Activities

MCP-1 and CCR2 are functionally similar in many aspects, but also some differences were observed. Both proteins are involved in mediating Th1/Th2 polarization. Generally, MCP-1 is associated with Th2 development in both infectious (Chensue

et al. 1996; Gu et al. 1997; Lu et al. 1998; Matsukawa et al. 2000) and allergic disease models (Gonzalo et al. 1998; Campbell et al. 1999). MCP-1 may promote Th2 development in the absence of CCR2 (Heesen et al. 1996; Luther and Cyster 2001; Traynor et al. 2002; Traynor and Huffnagle 2001). Response to *Schistosoma mansoni* eggs in MCP-$1^{-/-}$ mice revealed, however, that the defect caused by the absence of MCP-1 was not restricted to Th2 cytokines (Chensue et al. 1996; Lu et al. 1998). The study by Chensue et al. (1996) indicates that MCP-1 contributes more to type 2, but also to type 1 cytokine-mediated inflammation. The function of MCP-1 may depend on the stage and type of immune response (Matsukawa et al. 2000). CCR2, although may act together with MCP-1 in Th1 to Th2 switch, also promotes Th1 development in infectious models (Boring et al. 1997; Kurihara et al. 1997; Peters et al. 2000; Sato et al. 2000; Traynor et al. 2000, 2002; Peters et al. 2001) and attenuates the pulmonary allergic (Th2) response to *Aspergillus* (Blease et al. 2000).

MCP-1 may inhibit IL-12p40 production in monocytes, and IL-12p40 levels are correlated negatively with MCP-1 expression (Flores-Villanueva et al. 2005).

In a model of pulmonary *Cryptococcus neoformans* infection, MCP-1 neutralization and CCR2 deficiency similarly reduced cell recruitment to sites of infection and inflammation during the first 14 days of infection (macrophage recruitment was reduced by 54% and 65%, respectively; total lymphocyte recruitment – by 67% and 70%, respectively; CD4 T-cell recruitment – by 77% and 82%, respectively; CD8 T-cell recruitment – by 82% and 90%, respectively), caused significantly diminished IFN-gamma production by lung leukocytes, and increased IL-4 and IL-5 production by lung leukocytes (Th1 to Th2 switch) (Traynor et al. 2002). Enhancement of IL-4 and IL-5 production by T cells in response to MCP-1 was shown in other studies (Lukacs et al. 1997; Karpus et al. 1998). Recruitment of neutrophils and $B220^{+}$ cells was not significantly affected by either MCP-1 neutralization or by deletion of CCR2 under this infection (Traynor et al. 2002).

Although either MCP-1 neutralization or CCR2 deletion resulted in pulmonary *Cryptococcus neoformans* infection in increased production of IL-5, elevated levels of eotaxin (an eosinophil-selective chemoattractant whose actions are potentiated by IL-5 (Rothenberg 1999) in bronchoalveolar lavage fluid and eosinophil recruitment were observed only in CCR2 deficiency (Traynor et al. 2002). The CCR2 (but not MCP-1) expression was required for maximal expansion of the lung-associated lymph nodes with afferent Th1 development following pulmonary *Cryptococcus neoformans* infection (Traynor et al. 2002). The $CD4^{+}$ and $CD8^{+}$ T-cell numbers in the lung-associated lymph nodes of $CCR2^{-/-}$ mice were less than in the nodes of either $CCR2^{+/+}$ mice or MCP-1 neutralized mice (Traynor et al. 2002). CCR2, but not MCP-1, played a role in the recruitment of potential T1-promoting antigen-presenting cells to the pulmonary lymph nodes (Traynor et al. 2002). Expression of CCR2 was required for the development of Ag-specific interferon (IFN)-gamma-producing T cells in the lung-associated lymph nodes. In contrast, MCP-1 was not required for the development of Ag-specific IFN-gamma-producing T cells in the draining lymph nodes (Traynor et al. 2002).

Associations of MCP-1, CCR2, and CCR5 and Their Polymorphisms with Hepatitis

Activated hepatic stellate cells and to lesser extent Kupffer cells and endothelial cells are responsible for MCP-1 production in chronic liver disease (Czaja et al. 1994; Narumi et al. 1997; Marra et al. 1998). MCP-1 secretion is upregulated during chronic hepatitis. Expression analysis showed upregulation of MCP-1 in alcoholic hepatitis (6.2-fold), nonalcoholic steatohepatitis (5.8-fold), and hepatitis C (10.0-fold) compared to normal livers (Sarma et al. 2014). MCP-1 is a pro-inflammatory and pro-fibrotic chemokine, and its expression is strongly correlated to severity of liver inflammation and fibrosis (Marra et al. 1998).

Pharmacological inhibition of hepatic monocyte/macrophage infiltration by blocking MCP-1 during experimental liver damage ameliorated the development of hepatic steatosis (Baeck et al. 2012). Reduction of the intrahepatic expression of pro-inflammatory cytokines and chemokines, including MCP-1, tumor necrosis factor (TNF)-alpha, IFN-gamma, IL-1 beta, IL-6, IL-12, inducible nitric oxide synthase, integrin-alpha M, MIP-2, and chemokine (CXC motif) receptor 2 by emodin in concanavalin A-induced hepatitis protected mice from T-cell-mediated hepatitis, as shown by the decreased elevations of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as well as reduced hepatic necrosis (Xue et al. 2015). These recent data confirm the importance of pro-inflammatory cytokines and chemokines in hepatic injury.

Hepatitis C

Spontaneous Resolution or Persistence of HCV Infection

HCV infection may spontaneously resolve in approximately 20% of individuals (Micallef et al. 2006). In such cases, antibodies to HCV (anti-HCV) persist, but HCV ribonucleic acid (RNA) is not detected in the blood, and there are no signs or symptoms of HCV-associated liver disease. Human leukocyte antigen (HLA) DRB1*01 has been studied with regard to HCV spontaneous clearance with inconsistent results (Barrett et al. 1999; Fanning et al. 2000). Recent studies indicate that HCV clearance strongly depends on the *IFNL3*/*IFNL4* region on chromosome 19q13.13 (Prokunina-Olsson et al. 2013).

The *MCP1* polymorphism was also studied in respect to HCV clearance. In Tunisian HD patients, a possible role of the *MCP1* -2518 A/G variant in the spontaneous clearance or persistence of HCV infection was suggestive, but not statistically proven on the small study group (73 HD patients with persistently positive HCV RNA and 23 HD patients who have spontaneously eliminated HCV) (Ksiaa Cheikh Rouhou et al. 2011).

Among MCP-1 cell surface receptor genes, the promoter polymorphism of *CCR5* C-2132T was also found to be significantly, however weakly, associated with susceptibility to persistent HCV infection, with the presence of the C allele increasing the risk of persistent HCV carriage (Hellier et al. 2003). Table 2 summarizes the

results on associations of genes encoding the cell surface receptors that bind MCP-1 with spontaneous HCV resolution.

In the study by Mascheretti et al. (2004), variant allele p.Ile64 in the first transmembrane domain of *CCR2* was underrepresented in patients who had cleared HCV spontaneously. As CCR2 affinity to MCP-1 was shown to be unchanged despite the occurrence of this polymorphic variant (Lee et al. 1998), the association between p.64Ile and the lack of spontaneous recovery from HCV infection were explained in this study by LD of *CCR2* with the causative SNP, currently unknown (Mascheretti et al. 2004).

The allelic distribution of the *MCP1* -2518 G allele in combination with the *CCR2* 64Ile variant did not differ significantly between HD patients and controls or between patients with persistent HCV infection as assessed by two positive PCR tests for HCV RNA separated by more than 1 year and subjects who spontaneously recovered from HCV infection on the basis of two negative PCR tests for HCV RNA 1 year apart. The same applied for the other haplotype associations:

Table 2 Associations of genes encoding the cell surface receptors that bind MCP-1 with spontaneous HCV resolution

Study group	Association with spontaneous HCV resolution	Reference
CCR2 p.Val64Ile (*CCR2* G190A, rs1799864)		
Caucasians of German origin	Allele Ile64 was underrepresented in patients who had cleared HCV. This underrepresentation was not associated with female gender	Mascheretti (2004)
Caucasian women of Irish descent	Association not shown for HCV genotype 1b	Goulding et al. (2005)
Tunisian hemodialysis patients	Association not shown	Ksiaa Cheikh Rouhou et al. (2011)
CCR2 T780C (Asn260Asn)		
Caucasians of German origin	Association not shown	Mascheretti et al. (2004)
CCR5 C-2132T		
Caucasians, Afro-Caribbeans, Asians	Association of the C allele with persistent HCV carriage	Hellier et al. (2003)
CCR5-Δ32 (rs333)		
Caucasian women of Irish descent	Heterozygotes were more likely to have spontaneous clearance of HCV genotype 1b than those without the mutation	Goulding et al. (2005)
Tunisian hemodialysis patients	Association not shown	Ksiaa Cheikh Rouhou et al. (2011)
CCR5 -59029 A/G		
Tunisian hemodialysis patients	Association not shown	Ksiaa Cheikh Rouhou et al. (2011)

HCV hepatitis C virus, *MCP-1* monocyte chemotactic protein-1

CCR5-59029A/CCR2-64Ile, CCR5-59029A/CCR5-Δ32, and CCR5-Δ32/CCR2-64Ile (Ksiaa Cheikh Rouhou et al. 2011).

Persistent HCV Infection

HCV infection results in persistent HCV infection in approximately 80% of infected individuals (Micallef et al. 2006). Affected patients show positive HCV RNA testing. Individuals developing hepatitis C show positive HCV RNA testing and also abnormal liver enzyme activities for more than 6 months. HCV genotype 1 is the most prevalent worldwide, comprising 83.4 million cases (46.2% of all HCV cases), approximately one-third of which are in East Asia. Genotype 3 is the next most prevalent globally (54.3 million, 30.1%); genotypes 2, 4, and 6 are responsible for a total 22.8% of all cases; genotype 5 comprises the remaining <1% (Messina et al. 2015).

Persistence of HCV infection was associated with increased hepatic expression of MCP-1: MCP-1 mRNA was expressed in sinusoidal cells (Narumi et al. 1997), but not in liver-infiltrating lymphocytes (Leroy et al. 2003). Hepatic MCP-1 expression correlated with the severity of HCV-related liver disease (Narumi et al. 1997; Mühlbauer et al. 2003). On the other hand, Nischalke et al. (2004) found that intrahepatic mRNA levels of MCP-1 were markedly lower in chronic hepatitis C patients than in controls. This phenomenon had been explained by the inhibition of activity of the *MCP1* promoter by HCV core protein (Soo et al. 2002) and the downregulation of MCP-1 expression by viral proteins (Agnello et al. 1999). Recently, however, Sarma et al. (2014) have shown that HCV infection is associated with the downregulation of microRNA-107 (2.2-fold) and microRNA-449a (2.8-fold) in patients with HCV-mediated liver diseases which, by targeting components of the IL-6 receptor complex, resulted in increased hepatic MCP-1 expression. IL-6 receptor, JAK1, PU.1, and STAT3 are upregulated in HCV patients (Sarma et al. 2014). In HCV-infected patients, liver-infiltrating lymphocytes showed also increased expression of CCR5 (Shields et al. 1999).

The *MCP1* -2518 A/G polymorphism was associated with hepatic MCP-1 expression in hepatitis C. TNF-alpha-induced MCP-1 secretion of hepatic stellate cells isolated from carriers of the G allele was significantly higher than that from AA genotype possessing subjects (Mühlbauer et al. 2003).

The presence of HCV infection was not associated with *MCP-1* A-2076 T (Hellier et al. 2003), *CCR2* (p.Val64Ile) (Hellier et al. 2003), the Δ32 mutation in *CCR5* (Glas et al. 2003; Hellier et al. 2003), *CCR5*-2733 (Hellier et al. 2003), *CCR5*-2554 (Hellier et al. 2003), *CCR5*-2459 (Hellier et al. 2003), *CCR5* promoter 2135 (Hellier et al. 2003), *CCR5* promoter 2086 (Hellier et al. 2003), and *CCR5* promoter 1835 (Hellier et al. 2003).

The pathologic consequences of chronic HCV infection are liver inflammation and fibrosis.

Hepatic Inflammation

In the study by Narumi et al. (1997), serum levels of MCP-1 in patients with chronic persistent hepatitis C were elevated compared with those in normal volunteers, and

they were further significantly higher in patients with the active form of chronic hepatitis. In this study, elevated MCP-1 level was considered as a general phenomenon of inflammation, because it was elevated to the same extent in patients with rheumatoid arthritis (Narumi et al. 1997).

HCV-induced early IFN production by Kupffer cells triggers production of MCP-1. High levels of MCP-1 may recruit $CCR2^+$ leukocytes including monocytes, NK cells, and $CD4^+$ T cells into the liver to start inflammatory reactions (Matsuno et al. 2002).

In the general population of German origin, patients with chronic hepatitis C did not differ in a frequency distribution of *MCP1* -2518 A/G polymorphism from healthy subjects, but HCV-infected carriers of the G allele in *MCP1* -2518 A/G showed severe hepatic inflammation than the A allele bearers (Mühlbauer et al. 2003). Differences were the most apparent when comparing patients with no or only minimal portal inflammation without piecemeal necrosis (portal grading 0 or 1 (Desmet et al. 1994)) to patients with more severe portal inflammation (portal grading >1 (Desmet et al. 1994)) or comparing patients with no or only minimal lobular inflammation (lobular grading 0 or 1 (Desmet et al. 1994)) to patients with more severe lobular inflammation or necrosis (lobular grading >1 (Desmet et al. 1994; Mühlbauer et al. 2003). More advanced hepatic inflammation was associated with higher intrahepatic *MCP1* mRNA levels on the border of significance ($p = 0.055$) (Mühlbauer et al. 2003).

In the study by Hellier et al. (2003), there was a significant association between *CCR5*-Δ32 homozygotes and mild portal inflammation (a necroinflammatory score of <5 using the histology activity index (Desmet et al. 1994)), but *CCR5*-Δ32 was not associated with overall necroinflammatory score or interface hepatitis. Goulding et al. (2005) observed significantly lower hepatic inflammatory scores only for the *CCR5*-Δ32 heterozygote individuals who were simultaneously DRB1*03011 negative. HCV-infected patients representative for HCV-infected population in the United States who were carriers of *CCR5*-Δ32 (heterozygotes and homozygotes) had no significant differences in the degree of hepatic inflammation (mild inflammation defined as the histology activity index ≤ 5 vs. severe inflammation defined as the histology activity index >10) compared with those showing wild-type *CCR5* (Promrat et al. 2003). A significant difference was also not demonstrated when only whites were analyzed (Promrat et al. 2003).

Liver Fibrosis and Liver Cirrhosis Associated to Infection with HCV

About half of all HCV-infected patients progress from chronic hepatitis to liver cirrhosis (Poynard et al. 1997). Chemokines play important roles in HCV-induced liver fibrosis. HCV core protein NS5A has been found to increase reactive oxygen species (ROS) via mitochondrial insult (Korenaga et al. 2005). ROS activate Kupffer cells to release MCP-1 that transforms hepatic stellate cells into profibrogenic myofibroblasts that secrete alpha-smooth muscle actin and fibrillar collagens I and III. It has been suggested that MCP-1 may have a direct profibrogenic action via hepatic stellate cell chemotaxis (Marra et al. 1999). The relevance of MCP-1 in liver

fibrosis was supported by fibrosis regression with pharmacological inhibitor of MCP-1 in mice (Baeck et al. 2014).

Serum MCP-1 concentrations were significantly higher in HCV-infected subjects showing severe fibrosis compared to mild liver fibrosis (Lettow et al. 2011). Generally, carriers of the G allele at -2518 show higher serum MCP-1 levels than subjects bearing AA genotype (Rovin et al. 1999; Fenoglio et al. 2004; Flores-Villanueva et al. 2005; Buraczyńska et al. 2008; Xu et al. 2009). However, MCP-1 serum levels were not associated with *MCP1* -2518 A/G polymorphism in HCV-induced liver cirrhosis (Nahon et al. 2008). In the other study, mean level of *MCP1* expression in the whole blood of patients with HCV-associated liver cirrhosis was similar like in age- and sex-matched controls, although the cirrhotic patients showed higher frequency of the G allele than the controls did (Salama et al. 2014).

Carriers of the G allele in *MCP1* -2518 A/G were significantly more frequent in HCV patients with more advanced HCV-induced septal fibrosis or cirrhosis (stage 3 or 4 (Desmet et al. 1994)) than in those showing no or only mild periportal fibrosis (stage 1 or 2 (Desmet et al. 1994; Mühlbauer et al. 2003)). The estimated time to develop cirrhosis (stage 4 (Desmet et al. 1994)) was significantly shorter for carriers of the G allele in *MCP1* -2518 A/G than for AA homozygotes (Mühlbauer et al. 2003). In this study, patients who developed cirrhosis before the age of 50 years were defined as rapid fibrosers, whereas patients with stage 1 and a duration of infection of 10 years or longer were considered as slow fibrosers. Out of rapid fibrosers, 86% were carriers of G allele, whereas only 19% of slow fibrosers carried the G allele (Mühlbauer et al. 2003). More advanced hepatic fibrosis was associated with higher intrahepatic *MCP1* mRNA levels (Mühlbauer et al. 2003). The frequency of the G allele was also significantly higher in ascitic patients with post-hepatitis C liver cirrhosis than in controls, indicating that *MCP1* GG genotype and the G allele may predispose HCV-infected patients to a more progressive course of disease (Salama et al. 2014).

In a European cohort infected with HCV, a significant association was found between severe fibrosis (a fibrosis score of >3 using Ishak modification of the histology activity index (Ishak et al. 1995)) and carriage of the CCR5-Δ32 variant (Hellier et al. 2003). However, *CCR5*-Δ32 genotypes were similarly distributed in Caucasians of German origin showing mild–moderate fibrosis (stages 0, I, II according to the METAVIR system (Bedossa and Poynard 1996)) or severe fibrosis (stages III, IV according to the METAVIR system (Bedossa and Poynard 1996; Mascheretti et al. 2004)). HCV-infected patients representative for HCV-infected population in the United States who were carriers of *CCR5*-Δ32 (heterozygotes and homozygotes) had also no significant differences in the degree of hepatic fibrosis (mild fibrosis defined as Ishak score (Ishak et al. 1995) 0–2 vs. advanced fibrosis defined as Ishak score Ishak et al. 1995, 3–6) compared with those with wild-type *CCR5* (Promrat et al. 2003).

Genetic deletion of the MCP-1 receptor CCR2 resulted in reduced fibrosis in animal models of chronic liver damage (Karlmark et al. 2009; Seki et al. 2009). In a large European cohort, the *CCR2* polymorphism leading to p.Val64Ile substitution was not related to HCV-induced liver fibrosis (Hellier et al. 2003). Severity of liver

fibrosis was also not associated with *MCP1* 2076 (Hellier et al. 2003), *CCR5*-2733 (Hellier et al. 2003), *CCR5*-2554 (Hellier et al. 2003), *CCR5*-2459 (Hellier et al. 2003), *CCR5* promoter 2135 (Hellier et al. 2003), *CCR5* promoter 2132 (Hellier et al. 2003), *CCR5* promoter 2086 (Hellier et al. 2003), *CCR5* promoter 1835 (Hellier et al. 2003), and *DARC* polymorphism (Lettow et al. 2011).

As reported by Nahon et al. (2008), Child–Pugh score that employs clinical measures of liver disease (total bilirubin, serum albumin, prothrombin time, ascites, and hepatic encephalopathy) was not associated with *MCP1* -2518 A/G and *CCR5*-Δ 32 polymorphisms in HCV patients with liver cirrhosis (Nahon et al. 2008). The lack of association of *MCP1* -2518 A/G and *CCR5*-Δ32 polymorphisms with HCV genotype 1 frequency and death of cirrhotic HCV-infected patients was also demonstrated (Nahon et al. 2008). Baseline MCP-1 serum levels were not associated with the risk of death in this group as well (Nahon et al. 2008).

Patients with decompensated cirrhosis show higher susceptibility to bacterial infections (Vincent and Gustot 2010). Serum and ascitic fluid levels of IL-10 and MCP-1 were significantly higher in cirrhotic group with spontaneous bacterial peritonitis than in group without this complication (Kim et al. 2007). In ascitic patients with post-hepatitis C liver cirrhosis showing spontaneous bacterial peritonitis, the *MCP1* AG genotype occurred with higher frequency than in controls (76.0% vs. 40%, respectively) as well as in post-hepatitis C liver cirrhotic subjects without peritonitis (76.0% vs. 20%, respectively), suggesting that the *MCP1* AG genotype may increase the susceptibility to spontaneous bacterial peritonitis (Salama et al. 2014).

Hepatocellular Carcinoma Associated to Infection with HCV

Chemokines and their receptors can contribute to the pathogenesis of hepatocellular carcinoma, promoting proliferation of cancer cells, the inflammatory microenvironment of the tumor, evasion of the immune response, and angiogenesis (Marra and Tacke 2014). Mean time to occurrence of hepatocellular carcinoma in patients with HCV-related cirrhosis was 78.5 ± 40.5 months (Nahon et al. 2008). The lack of association of *MCP1* -2518 A/G (Nahon et al. 2008; Glas et al. 2004) and *CCR5*-Δ 32 (Nahon et al. 2008) polymorphisms with hepatocellular carcinoma occurrence was reported in cirrhotic HCV-infected patients.

Liver Enzymes

A significant positive correlation between the serum MCP-1 and aminotransferase levels suggested that serum MCP-1 levels could be related to the necroinflammatory activity of chronic hepatitis C (Narumi et al. 1997).

In majority of studies, serum ALT and/or AST activities were not associated with polymorphic variants of *MCP1* -2518 A/G (Nahon et al. 2008), *CCR2* (Val64Ile) (Goulding et al. 2005), or the Δ32 mutation in *CCR5* (Glas et al. 2003; Promrat et al. 2003; Goulding et al. 2005). In HCV-associated liver cirrhosis, lower ALT and AST activities were shown in heterozygotes or homozygotes for *CCR5*-Δ32 allele (Nahon et al. 2008).

HCV Load and Genotype

No significant correlation was found between intrahepatic MCP-1 levels and viral load or genotype (Nischalke et al. 2004). *MCP1* -2518 A/G polymorphism was also not associated with serum HCV RNA levels or frequencies of different HCV genotypes (Mühlbauer et al. 2003).

No significant differences in high ($>2 \times 10^6$ copies/mL) and low ($\leq 2 \times 10^6$ copies/mL) HCV titers were found in respect to *CCR2* (p.Val64Ile) (Promrat et al. 2003).

HCV-infected *CCR5*-Δ32 homozygotes had significantly higher HCV loads than wild-type patients (Woitas et al. 2002). The great majority of HCV-infected subjects tested in that study were HIV-seronegative white hemophiliacs. In a later study on Caucasians who tested negative for HIV infection, a significant difference of the HCV load was not associated with the Δ32 mutation in *CCR5* (Glas et al. 2003). HCV-infected patients representative for HCV-infected population in the United States who were carriers of *CCR5*-Δ32 (heterozygotes and homozygotes) had also no significant differences in high ($>2 \times 10^6$ copies/mL) and low ($\leq 2 \times 10^6$ copies/mL) HCV titers compared with those showing wild-type *CCR5* (Promrat et al. 2003). The *CCR5* promoter 59029 A allele was also not associated with viral load in that study (Promrat et al. 2003).

Response to Anti-HCV Therapy

Patients showing active HCV replication are treated with recombinant IFN-alpha monotherapy or receive combination therapy with IFN-alpha and ribavirin. Response to antiviral therapy was defined as being negative for HCV RNA and having normal liver function tests (normal activities of liver aminotransferases) after discontinuation of the antiviral therapy (end-of-treatment response) (Glas et al. 2003). Sustained response to antiviral therapy was defined as being negative for HCV RNA and having normal liver function tests (normal activities of liver aminotransferases) 24 weeks after discontinuation of the antiviral therapy (Hellier et al. 2003; Promrat et al. 2003; Mascheretti et al. 2004). Relapsers were those patients with non-detectable viremia during the course of treatment and detectable viremia following the end of drug administration (reappearance of HCV RNA after stopping the treatment) (Hellier et al. 2003). Nonresponders were defined as patients with continuous viremia more than 3 months into therapy (detectable HCV RNA during treatment) (Hellier et al. 2003). Nonresponse was also defined as the presence of HCV RNA 6 months after the end of treatment, including both relapsers and nonresponders (Promrat et al. 2003).

In 1997, better responsiveness to IFN therapy in chronic active hepatitis C was related to lesser grades of necroinflammatory activity and was predicted by the higher MCP-1 levels as well as higher serum MCP-1 to IFN-inducible protein-10 ratio (Narumi et al. 1997). However, in patients cured by IFN therapy, the MCP-1 levels only slightly decreased (Narumi et al. 1997). In the study by Nahon et al. (2008), sustained virological response was not dependent on baseline MCP-1 serum levels. In more recent study, pretreatment MCP-1 levels in patients with

sustained virological response after treatment with IFN and ribavirin were significantly lower than in nonresponders, and MCP-1 significantly decreased in patients with sustained response after 48 weeks of treatment (Gu et al. 2014).

The *MCP1* 2076 polymorphism was not associated with response to over 3 months of anti-HCV therapy (Hellier et al. 2003). The number of initial responders to IFN therapy (no HCV RNA detectable in serum 3 months after initiation of therapy) was not dependent on *MCP1* -2518 A/G as well (Mühlbauer et al. 2003). Sustained virological response was also not dependent on *MCP1* -2518 A/G (Nahon et al. 2008). Among non-Hispanic Caucasian patients treated with peginterferon and ribavirin after failing previous treatment with IFN, there were no significant associations between *MCP1* -2518 A/G and a sustained virological response (Morgan et al. 2008).

The polymorphic variant of *CCR2* (p.Val64Ile) was not associated with response to anti-HCV therapy in patients who had received treatment for more than 3 months (Hellier et al. 2003).

CCR5-Δ32 carriers had significantly lower end-of-treatment response rates than homozygous *CCR5* wild-type patients (10.5 vs. 39.0%) (Ahlenstiel et al. 2003), whereas sustained virological response rates showed a nonsignificant trend (5.3 vs. 18.6%) (Ahlenstiel et al. 2003) or were evidently nonsignificant (Nahon et al. 2008). Multivariate analysis confirmed *CCR5*-Δ32 carriage as an independent negative predictor for end-of-treatment response in IFN-alpha monotherapy (Ahlenstiel et al. 2003). In IFN-alpha/ribavirin-treated patients, *CCR5*-Δ32 carriers and *CCR5* wild-type patients had similar end-of-treatment response rates and sustained virological response rates (Ahlenstiel et al. 2003). Authors conclude that IFN-alpha/ribavirin combination treatment may overcome the negative effect of *CCR5*-Δ32 on results of antiviral treatment (Ahlenstiel et al. 2003). Carriage of the C allele at *CCR5*-2132 was also associated with an initial response to IFN, i.e., sustained responders and relapsers versus nonresponders, but no association was found in this study for *CCR5*-Δ32, *CCR5*-2733, *CCR5*-2554, *CCR5*-2459, *CCR5* promoter 2135, *CCR5* promoter 2086, and *CCR5* promoter 1835 (Hellier et al. 2003). In the study by Glas et al. (2003), *CCR5*-Δ32 was also not involved in response to 6 months of combination therapy with IFN-alpha-2a (3 × 3 million units per week) and ribavirin (1,000–1,200 mg daily according to body weight). Such a difference was also not observed when patients were stratified according to gender or HCV genotype (Glas et al. 2003). HCV-infected patients representative for HCV-infected population in the United States who were carriers of *CCR5*-Δ32 (heterozygotes and homozygotes) had no significant differences in response to antiviral therapy compared with those showing wild-type *CCR5* (Promrat et al. 2003). However, in multivariate logistic regression analysis controlling for factors known to affect treatment response (sex, race, viral genotype, and titer), there was a marginally ($p = 0.048$) positive association between *CCR5* promoter 59029 A allele carriers and sustained treatment response among HCV-infected subjects (Promrat et al. 2003).

No individual variants or *CCR2–CCR5* haplotypes appear to predict the likelihood of sustained response to antiviral therapy in Caucasians of German origin (Mascheretti et al. 2004).

Hepatitis B

Spontaneous HBV Resolution

About 90% of patients recover spontaneously after acute hepatitis B virus (HBV) infection, while about 10% of them fail to clear the virus and develop chronic infection (Fattovich 2003). The prevalence of persistence of HBV infection was found to be 100-fold higher in Asian (2.1%) than Caucasian (0.02%) subjects within the same community, suggesting an association between the genetic background and disease susceptibility (Kim et al. 2004). Patients who failed to clear the virus are at risk for developing end-stage liver disease and hepatocellular carcinoma. Host genetic factors related to the HLA system were shown to contribute to the course of HBV infection, including the ability to spontaneously eliminate viral DNA (Almarri and Batchelor 1994; Thursz et al. 1995). HLA allele DRB1*1302 was first identified as a protective factor against chronic HBV infection in Gambians (Thursz et al. 1995).

The role of *MCP1* -2518 A/G in HBV infection is not clear, although MCP-1 has been suggested to be a link in the chain involved in the hepatitis B outcome (Fierro et al. 2011; Meng et al. 2012). In the study by Park et al. (2006) on Korean subjects, the frequency of homozygotes for the *MCP1* -2518 A allele (A<G) among chronic HBV carrier patients was significantly higher than that among spontaneously recovered subjects. In multivariate analysis of Korean population, after adjustment for age and sex, there was, however, no significant difference in the frequencies of allele in the *MCP1* -2518, *CCR2* V64I, and *CCR5*-2459 polymorphisms between the HBV clearance group and the HBV persistence group (Cheong et al. 2007). Evidence that *CCR5* contributes to HBV persistence in Caucasians was provided by Thio et al. (2007). *CCR5*-Δ32, but not the A -2459G *CCR5* promoter variant or *CCR2* rs1799864, reduced the risk of developing a persistent HBV infection by nearly half (Thio et al. 2007).

Occult Hepatitis B

Individuals with occult hepatitis B – defined as the presence of HBV DNA in liver/serum with undetectable surface HBV antigen (HBsAg) – had significantly increased serum levels of MCP-1 compared to the healthy controls and patients that had resolved HBV infection [(HBsAg-negative, antibodies to HBV surface antigen (anti-HB)-positive)] (Fierro et al. 2011).

Chronic Hepatitis B

The MCP-1 expression level in the liver was higher in chronic hepatitis B complicated with nonalcoholic fatty liver disease than that shown in hepatitis B without such concomitant disease (Shen et al. 2012). No association of *MCP1* -2518, *CCR2* V64I, and *CCR5*-2459 SNPs with HBV disease progression was found (Cheong et al. 2007). In this analysis, inactive HBV carriers and chronic hepatitis B and HBV-associated liver cirrhotic patients (the progression group) were compared.

Hepatocellular Carcinoma Associated to Infection with HBV

MCP-1 was significantly upregulated in patients with hepatocellular carcinoma, showing HBV infection in over 50% of cases (Wang et al. 2013). These data indicate that higher MCP-1 level is generally associated with worse clinical condition in HBV infection. However, in the study by Park et al. (2006), there was no association between *MCP1* -2518 and development of hepatocellular carcinoma in the Korean population. The discrepancy between hepatic expression of MCP-1 and *MCP1* -2518 polymorphism may be related to downregulation of MCP-1 expression by viral proteins. MCP-1 expression was downregulated in HBV X protein transgenic cells (Agnello et al. 1999).

The development of anti-HBs usually follows HBsAg disappearance from the bloodstream, and spontaneous recovery from HBV infection is characterized by negative HBsAg and positive anti-HBs. A frequency distribution of *MCP1* polymorphic variants was, however, not associated with anti-HB development in response to HBV infection in HD patients, independent of diabetic status, but the *MCP1* -2518 G allele may predispose to HBsAg persistence (HBV carrier status) (Fig. 4) (Grzegorzewska et al. 2014). Stimulations with HBsAg and different fusion proteins eliciting moderate or high MCP-1 levels [with concomitant differences in TNF-alpha, IL-12, IL-10, IFN-gamma, and IL-6)] did not result in a significant difference in anti-HB levels in transgenic mice, and reductions in serum and liver HBsAg levels were dependent on stimulation (Meng et al. 2012). High-level productions of TNF-alpha and MCP-1 caused a more severe cytotoxicity in hepatocytes and were associated with less effective reduction of serum HBsAg level. Studies by Meng et al. (2012), although not exclusively related to MCP-1, clearly demonstrate that differences in MCP-1 concentrations do not correlate with anti-HB levels but may be important for HBsAg clearance. It has been suggested that the anti-HB response alone cannot account for the reduction of HBsAg (Meng et al. 2012), although anti-HB appearance in the bloodstream is usually associated with HBsAg clearance. Therefore, a lack of association between *MCP1* -2518 A/G and anti-HB development may not preclude the association between MCP-1 and HBV clearance indicated by HBsAg disappearance from the blood (Grzegorzewska et al. 2014).

The *CCR5*-59029 AA genotype was associated with an increased risk of chronic HBV infection, whereas the presence of the *CCR5*-59029 G allele was significantly associated with the spontaneous clearance of HBV (Chang et al. 2005).

Alcoholic Hepatitis

The frequency of -2518 G allele carriers did not differ between patients with alcoholic liver disease and healthy controls. However, more G allele carriers were shown in the severe alcoholic hepatitis patients than in other patients showing alcoholic liver disease (Degré et al. 2012). On the other hand, the lack of association of *MCP1* -2518 polymorphism with hepatocellular carcinoma

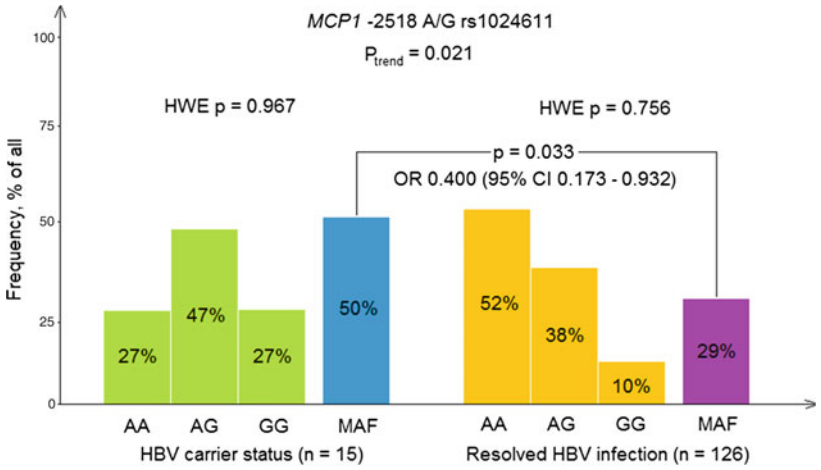

Fig. 4 Differences in a frequency distribution of *MCP1* -2518 A/G in hemodialysis patients who remained HBV carriers and those who spontaneously resolved HBV infection (Detailed results in Grzegorzewska et al. 2014)

occurrence was demonstrated in cirrhotic alcoholic patients (Nahon et al. 2007). *MCP1* -2518 G allele was not associated with 90-day survival of patients with alcoholic liver disease (Degré et al. 2012) or death of cirrhotic alcoholic patients (Nahon et al. 2007).

MCP-1 plasma levels are increased in alcoholic hepatitis patients compared with healthy subjects (Degré et al. 2012). Alcoholic hepatitis patients had significantly higher plasma levels and hepatic expression of MCP-1 than alcoholic patients without alcoholic hepatitis. Downregulation of microRNA-107 or microRNA-449a was not shown in patients with alcoholic hepatitis (Sarma et al. 2014). Plasma levels and hepatic expression of MCP-1 were associated with alcoholic hepatitis severity. MCP-1 liver expression was correlated with neutrophil infiltrate and IL-8 expression, but not with steatosis. However, higher MCP-1 plasma levels or higher MCP-1 liver expression in G allele carriers were not found (Degré et al. 2012). Baseline MCP-1 serum levels were associated neither with the risk of death nor with the risk of hepatocellular carcinoma in cirrhotic alcoholic patients Nahon et al. 2007.

In an animal model, MCP-1 played a role in alcoholic liver injury independently of CCR2 (Mandrekar et al. 2011). In a human study, a frequency distribution of *CCR2* 190 A/G polymorphism did not yield significant differences between alcoholic liver disease patients and controls, patients with or without alcoholic liver cirrhosis, and patients with or without alcoholic hepatitis (Degré et al. 2012). Severity of alcoholic hepatitis did not influence statistical analysis (Degré et al. 2012). There were no associations of *CCR2*-64I and *CCR5*-Δ32 polymorphisms with hepatocellular carcinoma occurrence in cirrhotic alcoholic patients Nahon et al. 2007.

Nonalcoholic Steatohepatitis

In nonalcoholic steatohepatitis, both MCP-1 and CCR2 levels are upregulated, causing macrophage infiltration resulting in inflammation, fibrosis, and steatosis (Marra and Tacke 2014). Downregulation of microRNA-107 or microRNA-449a was not shown in patients with nonalcoholic steatohepatitis (Sarma et al. 2014).

Potential Applications to Prognosis, Other Diseases or Conditions

Potential application of MCP-1, receptors of MCP-1, and polymorphisms of their genes to prognosis of hepatitis is shown in Table 3. Gathered evidence shows that awareness of *MCP1* -2518 A/G and *CCR5* SNPs among hepatitis patients may have prognostic value in regard to hepatitis course and treatment responsiveness. Results indicate that serum/plasma MCP-1 levels can be suggestive for hepatitis occurrence in HBV-infected patients and alcoholic subjects. It is worthy to examine hepatic tissue specimens for MCP-1 mRNA to better establish severity of liver disease. Tested *CCR2* polymorphisms were not predictive either for resolution of viral infections or hepatitis severity; therefore their application to clinical practice in this respect cannot be advisable.

MCP1 -2518 A/G polymorphism was also associated with several other diseases, like pulmonary tuberculosis (Flores-Villanueva et al. 2005; Xu et al. 2009, Ben-Selma et al. 2011), ischemic stroke (Flex et al. 2004; Uchida et al. 2012), type 1 diabetes (Yang et al. 2004), type 2 diabetes (Karadeniz et al. 2010), coronary artery disease (Szalai et al. 2001a), bronchial asthma (Szalai et al. 2001b), and Crohn's disease (Herfarth et al. 2003). Associations of *MCP1* -2518 A/G polymorphism with primary glomerulonephritis (Mostowska et al. 2012), Alzheimer's disease (Fenoglio et al. 2004), and systemic lupus erythematosus (Aguilar et al. 2001) were not shown, although urinary MCP-1 was associated with activity lupus nephropathy (Kiani et al. 2009).

Summary Points

1. The *MCP1* -2518 G allele is associated with upregulation of monocyte chemotactic protein-1 (MCP-1) having a significant role in leukocyte recruitment and trafficking.
2. The cell surface receptors that bind MCP-1 are CCR2 and CCR5 encoded by genes (*CCR2* and *CCR5*, respectively) located on the chromosome 3p21.
3. Associations of MCP-1, CCR2, and CCR5 and their polymorphisms with hepatitis were explored in several studies, usually designed as cross-sectional or observational.
4. In HCV infection, heterozygotes in *CCR5*-Δ32 (rs333) were more likely to have spontaneous clearance of HCV genotype 1b, hepatic MCP-1 expression correlated with severity of HCV-related liver disease, and was related to *MCP1* -2518

Table 3 Potential application of MCP-1, receptors for MCP-1, and polymorphisms of their genes to prognosis of hepatitis

Parameter	Patient relevant for testing	Diagnostic value	Reference
Polymorphisms			
MCP1 -2518 A/G (risk G allele)	Hepatitis C, alcoholic hepatitis	In hepatitis C: indication of hepatic MCP-1 expression; prognosis of hepatic fibrosis and ascites; indication of hepatitis course severity	Mühlbauer et al. (2003), Degré et al. (2012), and Salama et al. (2014)
		Indication of severity of alcoholic hepatitis	
CCR5 C-2132T (favorable C allele)	Hepatitis C	Prediction of treatment response	Hellier et al. (2003)
CCR5-Δ32 (rs 333)	Hepatitis C, hepatitis B	Prediction of HCV and HBV resolution	Goulding et al. (2005), Ahlenstiel (2003), and Thio et al. (2007)
		Prediction of IFN-alpha end-of-treatment response in hepatitis C	
CCR5-59029 A/G	Hepatitis C, hepatitis B	Prediction of sustained response to treatment with IFN in hepatitis C	Promrat et al. (2003) and Chang (2005)
		Prediction of HBV persistence	
Serum measurements			
MCP-1	Hepatitis C, hepatitis B, alcoholic hepatitis	Nonspecific indication of hepatic necroinflammatory severity in hepatitis C	Narumi et al. (1997), Fierro (2011), Degré (2012), and Sarma et al. (2014)
		Indication of probability of occult hepatitis B and alcoholic hepatitis	
		Indication of severity of alcoholic hepatitis	
Hepatic tissue specimens			
MCP-1 mRNA	Hepatitis C, alcoholic hepatitis, nonalcoholic steatohepatitis	Indication of severity of liver disease	Narumi et al. (1997), Mühlbauer et al. (2003), and Sarma et al. (2014)
		Indication of hepatic fibrosis	

HBV hepatitis B virus, *HCV* hepatitis C virus, *IFN* interferon, *MCP-1* monocyte chemotactic protein-1, *mRNA* messenger ribonucleic acid

A/G polymorphism; *CCR5* C-2132T, *CCR5*-59029 A/G, and *CCR5*-Δ32 were associated with prediction of treatment response.

5. In HBV infection, *CCR5*-Δ32 (rs333) and *CCR5*-59029 A/G were associated with prediction of HBV resolution or persistence.
6. In alcoholic hepatitis, the *MCP1* -2518 G allele and MCP-1 expression in hepatic tissue specimens indicated severity of disease.

7. In nonalcoholic steatohepatitis, MCP-1 expression in hepatic tissue correlated with hepatic fibrosis and severity of liver disease.

References

Agnello V, Abel G, Elfahal M, Knight GB, Zhang QX. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc Natl Acad Sci U S A. 1999;96:12766–71.

Aguilar F, González-Escribano MF, Sánchez-Román J, Núñez-Roldán A. MCP-1 promoter polymorphism in Spanish patients with systemic lupus erythematosus. Tissue Antigens. 2001; 58(5):335–8.

Ahlenstiel G, Berg T, Woitas RP, Grünhage F, Iwan A, Hess L, Brackmann HH, Kupfer B, Schernick A, Sauerbruch T, Spengler U. Effects of the CCR5-Delta32 mutation on antiviral treatment in chronic hepatitis C. Hepatology. 2003;39:245–52.

Algood HM, Flynn JL. CCR5-deficient mice control *Mycobacterium tuberculosis* infection despite increased pulmonary lymphocytic infiltration. J Immunol. 2004;173:3287–96.

Almarri A, Batchelor JR. HLA and hepatitis B infection. Lancet. 1994;344:1194–5.

Andres PG, Beck PL, Mizoguchi E, Bhan AK, Dawson T, Kuziel WA, Maeda N, MacDermott RP, Podolsky DK, Reinecker HC. Mice with a selective deletion of the CC chemokine receptors 5 or 2 are protected from dextran sodium sulfate-mediated colitis: lack of CC chemokine receptor 5 expression results in an NK1.1 lymphocyte-associated Th2-type immune response in the intestine. J Immunol. 2000;164:6303–12.

Ansel KM, Mc Heyzer-Willaims LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG. In vivo activated CD4T cells upregulate CXC chemokine receptor 5 and reprogramme their response to lymphoid chemokines. J Exp Med. 1999;190:1123–34.

Baeck C, Wehr A, Karlmark KR, Heymann F, Vucur M, Gassler N, Huss S, Klussmann S, Eulberg D, Luedde T, Trautwein C, Tacke F. Pharmacological inhibition of the chemokine CCL2 (MCP-1) diminishes liver macrophage infiltration and steatohepatitis in chronic hepatic injury. Gut. 2012;61:416–26.

Baeck C, Wei X, Bartneck M, Fech V, Heymann F, Gassler N, Hittatiya K, Eulberg D, Luedde T, Trautwein C, Tacke F. Pharmacological inhibition of the chemokine C-C motif chemokine ligand 2 (monocyte chemoattractant protein 1) accelerates liver fibrosis regression by suppressing Ly-6C(+) macrophage infiltration in mice. Hepatology. 2014;59:1060–72.

Barrett S, Ryan E, Crowe J. Association of the HLA-DRB1*01 allele with spontaneous viral clearance in an Irish cohort infected with hepatitis C virus via contaminated anti-D immunoglobulin. J Hepatol. 1999;30:979–83.

Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology. 1996;24:289–93.

Ben-Selma W, Harizi H, Boukadida J. MCP-1 -2518 A/G functional polymorphism is associated with increased susceptibility to active pulmonary tuberculosis in Tunisian patients. Mol Biol Rep. 2011;38:5413–9.

Blease K, Mehrad B, Standiford TJ, Lukacs NW, Gosling J, Boring L, Charo IF, Kunkel SL, Hogaboam CM. Enhanced pulmonary allergic responses to Aspergillus in $CCR2^{-/-}$ mice. J Immunol. 2000;165:2603–11.

Boring L, Gosling J, Chensue SW, Kunkel SL, Farese Jr RV, Broxmeyer HE, Charo IF. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. J Clin Invest. 1997;100:2552–61.

Buraczyńska M, Bednarek-Skublewska A, Buraczyńska K, Książek A. Monocyte chemoattractant protein-1 (MCP-1) gene polymorphism as a potential risk factor for cardiovascular disease in hemodialyzed patients. Cytokine. 2008;44:361–5.

Campbell EM, Charo IF, Kunkel SL, Strieter RM, Boring L, Gosling J, Lukacs NW. Monocyte chemoattractant protein-1 mediates cockroach allergen-induced bronchial hyperreactivity in normal but not CCR2$^{-/-}$ mice: the role of mast cells. J Immunol. 1999;163:2160–7.

Chang HY, Ahn SH, Kim DY, Shin JS, Kim YS, Hong SP, Chung HJ, Kim SO, Yoo WD, Han KH. Association between CCR5 promoter polymorphisms and hepatitis B virus infection [Article in Korean]. Korean J Hepatol. 2005;11:116–24.

Chensue SW, Warmington KS, Ruth JH, Sanghi PS, Lincoln P, Kunkel SL. Role of monocyte chemoattractant protein-1 (MCP-1) in Th1 (mycobacterial) and Th2 (schistosomal) antigen-induced granuloma formation: relationship to local inflammation, Th cell expression, and IL-12 production. J Immunol. 1996;157:4602–8.

Cheong JY, Cho SW, Choi JY, Lee JA, Kim MH, Lee JE, Hahm KB, Kim JH. RANTES, MCP-1, CCR2, CCR5, CXCR1 and CXCR4 gene polymorphisms are not associated with the outcome of hepatitis B virus infection: results from a large scale single ethnic population. J Korean Med Sci. 2007;22:529–35.

Czaja MJ, Geerts A, Xu J, Schmiedeberg P, Ju Y. Monocyte chemoattractant protein 1 (MCP-1) expression occurs in toxic rat liver injury and human liver disease. J Leuk Biol. 1994;55:120–6.

Degré D, Lemmers A, Gustot T, Ouziel R, Trépo E, Demetter P, Verset L, Quertinmont E, Vercruysse V, Le Moine O, Devière J, Moreno C. Hepatic expression of CCL2 in alcoholic liver disease is associated with disease severity and neutrophil infiltrates. Clin Exp Immunol. 2012;169:302–10.

Desmet V, Gerber M, Hoofnagle JH, Manns M, Scheuer P. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology. 1994;19:1513–20.

Fanning LJ, Levis J, Kenny-Walsh E, Wynne F, Whelton M, Shanahan F. Viral clearance in hepatitis C (1b) infection: relationship with human leukocyte antigen class II in a homogeneous population. Hepatology. 2000;31:1334–7.

Fattovich G. Natural history and prognosis of hepatitis B. Semin Liver Dis. 2003;23:47–58.

Fenoglio C, Galimberti D, Lovati C, Guidi I, Gatti A, Fogliarino S, Tiriticco M, Mariani C, Forloni G, Pettenati C, Baron P, Conti G, Bresolin N, Scarpini E. MCP-1 in Alzheimer's disease patients: a -2518G polymorphism and serum levels. Neurobiol Aging. 2004;25:1169–73.

Fierro NA, Roman S, Realpe M, Hernandez-Nazara Z, Zepeda-Carrillo EA, Panduro A. Multiple cytokine expression profiles reveal immune-based differences in occult hepatitis B genotype H-infected Mexican Nahua patients. Mem Inst Oswaldo Cruz. 2011;106:1007–13.

Flex A, Gaetani E, Papaleo P, Straface G, Proia AS, Pecorini G, Tondi P, Pola P, Pola R. Proinflammatory genetic profiles in subjects with history of ischemic stroke. Stroke. 2004;35:2270–5.

Flores-Villanueva PO, Ruiz-Morales JA, Song CH, Flores LM, Jo EK, Montaño M, Barnes PF, Selman M, Granados J. A functional promoter polymorphism in monocyte chemoattractant protein-1 is associated with increased susceptibility to pulmonary tuberculosis. J Exp Med. 2005;202:1649–58.

Franci C, Wong LM, Van DJ, Proost P, Charo IF. Monocyte chemoattractant protein–3, but not monocyte chemoattractant protein–2, is a functional ligand of the human monocyte chemoattractant protein–1 receptor. J Immunol. 1995;154:6511–7.

Glas J, Török HP, Simperl C, König A, Martin K, Schmidt F, Schaefer M, Schiemann U, Folwaczny C. The Delta 32 mutation of the chemokine-receptor 5 gene neither is correlated with chronic hepatitis C nor does it predict response to therapy with interferon-alpha and ribavirin. Clin Immunol. 2003;108:46–50.

Glas J, Török HP, Tonenchi L, Schiemann U, Folwaczny C. The -2518 promoter polymorphism in the MCP-1 gene is not associated with liver cirrhosis in chronic hepatitis C virus infection. Gastroenterology. 2004;126:1930–1.

Gonzalo JA, Lloyd CM, Wen D, Albar JP, Wells TN, Proudfoot A, Martinez AC, Dorf M, Bjerke T, Coyle AJ, Gutierrez-Ramos JC. The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. J Exp Med. 1998;188:157–67.

Goulding C, Murphy A, MacDonald G, Barrett S, Crowe J, Hegarty J, McKiernan S, Kelleher D. The CCR5-D32 mutation: impact on disease outcome in individuals with hepatitis C infection from a single source. Gut. 2005;54:1157–61.

Grzegorzewska AE, Pajzderski D, Sowińska A, Jagodziński PP. Monocyte chemoattractant protein-1 gene (MCP-1-2518 A/G) polymorphism and serological markers of hepatitis B virus infection in hemodialysis patients. Med Sci Monit. 2014;20:1101–16.

Gu L, Rutledge B, Fiorillo J, Ernst C, Grewal I, Flavell R, Gladue R, Rollins B. In vivo properties of monocyte chemoattractant protein-1. J Leukoc Biol. 1997;62:577–80.

Gu B, Ye B, Mao WL, Ye JL. Monocyte chemotactic protein-1 as possible prognostic markers of the efficacy of antiviral treatment in chronic hepatitis C. Hepatogastroenterology. 2014;61:55–8.

Heesen M, Tanabe S, Berman MA, Yoshizawa I, Luo Y, Kim RJ, Post TW, Gerard C, Dorf ME. Mouse astrocytes respond to the chemokines MCP-1 and KC, but reverse transcriptase-polymerase chain reaction does not detect mRNA for the KC or new MCP-1 receptor. J Neurosci Res. 1996;45:382–91.

Hellier S, Frodsham AJ, Hennig BJ, Klenerman P, Knapp S, Ramaley P, Satsangi J, Wright M, Zhang L, Thomas HC, Thursz M, Hill AV. Association of genetic variants of the chemokine receptor CCR5 and its ligands, RANTES and MCP-2, with outcome of HCV infection. Hepatology. 2003;38:1468–76.

Herfarth H, Göke M, Hellerbrand C, Mühlbauer M, Vogl D, Schölmerich J, Rogler G. Polymorphism of monocyte chemoattractant protein 1 in Crohn's disease. Int J Colorectal Dis. 2003;18:401–5.

Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN, et al. Histological grading and staging of chronic hepatitis. J Hepatol. 1995;22:696–9.

Karadeniz M, Erdogan M, Cetinkalp S, Berdeli A, Eroglu Z, Ozgen AG. Monocyte chemoattractant protein-1 (MCP-1) 2518G/A gene polymorphism in Turkish type 2 diabetes patients with nephropathy. Endocrine. 2010;37:513–7.

Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, Merad M, Luedde T, Trautwein C, Tacke F. Hepatic recruitment of the inflammatory Gr1 monocyte subset upon liver injury promotes hepatic fibrosis. Hepatology. 2009;50:261–74.

Karpus WJ, Kennedy KJ, Kunkel SL, Lukacs NW. Monocyte chemotactic protein 1 regulates oral tolerance induction by inhibition of T helper cell 1-related cytokines. J Exp Med. 1998;187:733–41.

Kiani AN, Johnson K, Chen C, Diehl E, Hu H, Vasudevan G, Singh S, Magder LS, Knechtle SJ, Petri M. Urine osteoprotegerin and monocyte chemoattractant protein-1 in lupus nephritis. J Rheumatol. 2009;36:2224–30.

Kim WR, Benson JT, Therneau TM, Torgerson HA, Yawn BP, Melton III LJ. Changing epidemiology of hepatitis B in a U.S. community. Hepatology. 2004;39:811–6.

Kim MS, Day CJ, Morrison NA. MCP-1 is induced by receptor activator of nuclear factor-{kappa} B ligand, promotes human osteoclast fusion, and rescues granulocyte macrophage colony-stimulating factor suppression of osteoclast formation. J Biol Chem. 2005;280:16163–9.

Kim JK, Chon CY, Kim JH, Kim YJ, Cho JH, Bang SM, Ahn SH, Han KH, Moon YM. Changes in serum and ascitic monocyte chemotactic protein-1 (MCP-1) and IL-10 levels in cirrhotic patients with spontaneous bacterial peritonitis. J Interferon Cytokine Res. 2007;27:227–30.

Kolattukudy PE, Niu J. Inflammation, endoplasmic reticulum stress, autophagy, and the monocyte chemoattractant protein-1/CCR2 pathway. Circ Res. 2012;110:174–89.

Korenaga M, Wang T, Li Y, Showalter LA, Chan T, Sun J, Weinman SA. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. J Biol Chem. 2005;280:37481–8.

Ksiaa Cheikh Rouhou L, Gorgi YL, Skhiri HA, Aouadi H, Ayed SJ, Sfar I, Ayed K, Ben Abdallah T. Chemokine and chemokine receptor gene polymorphism in Tunisian hemodialysis patients with HCV infection. Arab J Nephrol Transplant. 2011;4:117–24.

Kurihara T, Warr G, Loy J, Bravo R. Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. J Exp Med. 1997;186:1757–62.

Lee B, Doranz BJ, Rana S, Yi Y, Mellado M, Frade JM, Martinez-A C, O'Brien SJ, Dean M, Collman RG, Doms RW. Influence of the CCR2-V64I polymorphism on human immunodeficiency virus type 1 coreceptor activity and on chemokine receptor function of CCR2b, CCR3, CCR5, and CXCR4. J Virol. 1998;72:7450–8.

Leroy V, Vigan I, Mosnier JF, Dufeu-Duchesne T, Pernollet M, Zarski JP, Marche PN, Jouvin-Marche E. Phenotypic and functional characterization of intrahepatic T lymphocytes during chronic hepatitis C. Hepatology. 2003;38:829–41.

Lettow I, Berres ML, Schmitz P, Müller T, Berg T, Neumann UP, Trautwein C, Wasmuth HE. A Duffy antigen receptor for chemokines (DARC) polymorphism that determines pro-fibrotic chemokine serum concentrations is not directly associated with severity of hepatitis C infection. Hum Immunol. 2011;72:273–7.

Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, MacDonald ME, Stuhlmann H, Koup RA, Landau NR. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply exposed individuals to HIV-1 infection. Cell. 1996;86:367–77.

Lu B, Rutledge BJ, Gu L, Fiorillo J, Lukacs NW, Kunkel SL, North R, Gerard C, Rollins BJ. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. J Exp Med. 1998;187:601–8.

Lukacs NW, Chensue SW, Karpus WJ, Lincoln P, Keefer C, Strieter RM, Kunkel SL. C-C chemokines differentially alter interleukin-4 production from lymphocytes. Am J Pathol. 1997;150:1861–8.

Luther SA, Cyster JG. Chemokines as regulators of T cell differentiation. Nat Immunol. 2001;2:102–7.

Mandrekar P, Ambade A, Lim A, Szabo G, Catalano D. An essential role for MCP-1 in alcoholic liver injury: regulation of pro-inflammatory cytokines and hepatic steatosis. Hepatology. 2011;54:2185–97.

Mantovani A, Bonecchi R, Locati M. Tuning inflammation and immunity by chemokine sequestration: decoys and more. Nat Rev Immunol. 2006;6:907–18.

Marra F, Tacke F. Roles for chemokines in liver disease. Gastroenterology. 2014;147:577–94e1.

Marra F, DeFranco R, Grappone C, Milani S, Pastacaldi S, Pinzani M, Romanelli RG, Laffi G, Gentilini P. Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration. Am J Pathol. 1998;152:423–30.

Marra F, Romanelli RG, Giannini C, Failli P, Pastacaldi S, Arrighi MC, Pinzani M, Laffi G, Montalto P, Gentilini P. Monocyte chemotactic protein-1 as a chemoattractant for human hepatic stellate cells. Hepatology. 1999;29:140–8.

Mascheretti S, Hinrichsen H, Ross S, Buggisch P, Hampe J, Foelsch UR, Schreiber S. Genetic variants in the CCR gene cluster and spontaneous viral elimination in hepatitis C-infected patients. Clin Exp Immunol. 2004;136:328–33.

Matsukawa A, Lukacs NW, Standiford TJ, Chensue SW, Kunkel SL. Adenoviral-mediated overexpression of monocyte chemoattractant protein-1 differentially alters the development of Th1 and Th2 type responses in vivo. J Immunol. 2000;164:1699–704.

Matsuno K, Nomiyama H, Yoneyama H, Uwatoku R. Kupffer cell-mediated recruitment of dendritic cells to the liver crucial for a host defense. Dev Immunol. 2002;9:143–9.

McDermott DH, Zimmerman PA, Guignard F, Kleeberger CA, Leitman SF, Murphy PM. CCR5 promoter polymorphism and HIV-1 disease progression. Multicenter AIDS Cohort Study (MACS). Lancet. 1998;352:866–70.

Meng ZF, Wang HJ, Yao X, Wang XY, Wen YM, Dai JX, Xie YH, Xu JQ. Immunization with HBsAg-Fc fusion protein induces a predominant production of Th1 cytokines and reduces HBsAg level in transgenic mice. Chin Med J (Engl). 2012;125:3266–72.

Messina JP, Humphreys I, Flaxman A, Brown A, Cooke GS, Pybus OG, Barnes E. Global distribution and prevalence of hepatitis C virus genotypes. Hepatology. 2015;61:77–87.

Micallef JM, Kaldor JM, Dore GJ. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. J Viral Hepat. 2006;13:34–41.

Morgan TR, Lambrecht RW, Bonkovsky HL, Chung RT, Naishadham D, Sterling RK, Fontana RJ, Lee WM, Ghany MG, Wright EC, O'Brien TR, Trial Group HALT-C. DNA polymorphisms and response to treatment in patients with chronic hepatitis C: results from the HALT-C trial. J Hepatol. 2008;49:548–56.

Mostowska M, Lianeri M, Oko A, Mostowska A, Jagodziński PP. No association of monocyte chemoattractant protein-1 -2518 A/G polymorphism with the risk of primary glomerulonephritis in the Polish population. Mol Biol Rep. 2012;39:5933–41.

Mühlbauer M, Bosserhoff AK, Hartmann A, Thasler WE, Weiss TS, Herfarth H, Lock G, Schölmerich J, Hellerbrand C. A novel MCP-1 gene polymorphism is associated with hepatic MCP-1 expression and severity of HCV-related liver disease. Gastroenterology. 2003;125:1085–93.

Nahon P, Sutton A, Rufat P, Faisant C, Simon C, Barget N, Trinchet JC, Beaugrand M, Gattegno L, Charnaux N. Lack of association of some chemokine system polymorphisms with the risks of death and hepatocellular carcinoma occurrence in patients with alcoholic cirrhosis: a prospective study. Eur J Gastroenterol Hepatol. 2007;19:425–31.

Nahon P, Sutton A, Rufat P, Simon C, Trinchet JC, Gattegno L, Beaugrand M, Charnaux N. Chemokine system polymorphisms, survival and hepatocellular carcinoma occurrence in patients with hepatitis C virus-related cirrhosis. World J Gastroenterol. 2008;14:713–9.

Narumi S, Tominaga Y, Tamaru M, Shimai S, Okumura H, Nishioji K, Itoh Y, Okanoue T. Expression of IFN-inducible protein-10 in chronic hepatitis. J Immunol. 1997;158:5536–44.

Nischalke HD, Nattermann J, Fischer HP, Sauerbruch T, Spengler U, Dumoulin FL. Semiquantitative analysis of intrahepatic CC-chemokine mRNas in chronic hepatitis C. Mediators Inflamm. 2004;13:357–9.

Nomiyama H, Hieshima K, Nakayama T, Sakaguchi T, Fujisawa R, Tanase S, Nishiura H, Matsuno K, Takamori H, Tabira Y, Yamamoto T, Miura R, Yoshie O. Human CC chemokine liver-expressed chemokine/CCL16 is a functional ligand for CCR1, CCR2 and CCR5, and constitutively expressed by hepatocytes. Int Immunol. 2001;13:1021–9.

Papayianni A, Alexopoulos E, Giamalis P, Gionanlis L, Belechri AM, Koukoudis P, Memmos D. Circulating levels of ICAM-1, VCAM-1, and MCP-1 are increased in haemodialysis patients: association with inflammation, dyslipidaemia, and vascular events. Nephrol Dial Transplant. 2002;17:435–41.

Park BL, Kim YJ, Cheong HS, Kim LH, Choi YH, Lee HS, Shin HD. Association of common promoter polymorphisms of MCP1 with hepatitis B virus clearance. Exp Mol Med. 2006;38:694–702.

Peters W, Dupuis M, Charo IF. A mechanism for the impaired IFN-γ productioninC-C chemokine receptor 2 (CCR2) knockout mice: role of CCR2 in linking the innate and adaptive immune responses. J Immunol. 2000;165:7072–7.

Peters W, Scott HM, Chambers HF, Flynn JL, Charo IF, Ernst JD. Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis. Proc Natl Acad Sci U S A. 2001;98:7958–63.

Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet. 1997;349:825–32.

Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, Hergott D, Porter-Gill P, Mumy A, Kohaar I, Chen S, Brand N, Tarway M, Liu L, Sheikh F, Astemborski J, Bonkovsky HL, Edlin BR, Howell CD, Morgan TR, Thomas DL, Rehermann B, Donnelly RP, O'Brien TR. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet. 2013;45:164–71.

Promrat K, McDermott DH, Gonzalez CM, Kleiner DE, Koziol DE, Lessie M, Merrell M, Soza A, Heller T, Ghany M, Park Y, Alter HJ, Hoofnagle JH, Murphy PM, Liang TJ. Associations of

chemokine system polymorphisms with clinical outcomes and treatment responses of chronic hepatitis C. Gastroenterology. 2003;124:352–60.
Rollins BJ. Monocyte chemoattractant protein 1: a potential regulator of monocyte recruitment in inflammatory disease. Mol Med Today. 1996;2:198–204.
Rollins BJ, Walz A, Baggiolini M. Recombinant human MCP-1/JE induces chemotaxis, calcium flux, and the respiratory burst in human monocytes. Blood. 1991;78:1112–6.
Rothenberg ME. Eotaxin: an essential mediator of eosinophil trafficking into mucosal tissues. Am J Respir Cell Mol Biol. 1999;21:291–5.
Rovin BH, Lu L, Saxena R. A novel polymorphism in the MCP-1 gene regulatory region that influences MCP-1 expression. Biochem Biophys Res Commun. 1999;259:344–8.
Salama MK, Sabry D, Al-Ghussein MA, Ahmed R, AbdAllah S, Taha FM, Fathy W, Wadie MS, Nabih M, Abul-Fotouh A, Darwish T. Molecular detection of monocyte chemotactic protein-1 polymorphism in spontaneous bacterial peritonitis patients. World J Gastroenterol. 2014; 20(33):11793–9.
Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J Exp Med. 1998;187:875–83.
Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M. Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature. 1996;382:722–5.
Sarafi MN, Garcia-Zepeda EA, MacLean JA, Charo IF, Luster AD. Murine monocyte chemoattractant protein (MCP)–5: a novel CC chemokine that is a structural and functional homologue of human MCP-1. J Exp Med. 1997;185:99–109.
Sarma NJ, Tiriveedhi V, Crippin JS, Chapman WC, Mohanakumar T. Hepatitis C virus-induced changes in microRNA 107 (miRNA-107) and miRNA-449a modulate CCL2 by targeting the interleukin-6 receptor complex in hepatitis. J Virol. 2014;88:3733–43.
Sato N, Ahuja SK, Quinones M, Kostecki V, Reddick RL, Melby PC, Kuziel WA, Ahuja SS. CC chemokine receptor (CCR)2 is required for langerhans cell migration and localization of T helper cell type 1 (Th1)-inducing dendritic cells: absence of CCR2 shifts the Leishmania major-resistant phenotype to a susceptible state dominated by Th2 cytokines, B cell outgrowth, and sustained neutrophilic inflammation. J Exp Med. 2000;192:205–18.
Schnabel RB, Baumert J, Barbalic M, Dupuis J, Ellinor PT, Durda P, Dehghan A, Bis JC, Illig T, Morrison AC, Jenny NS, Keaney Jr JF, Gieger C, Tilley C, Yamamoto JF, Khuseyinova N, Heiss G, Doyle M, Blankenberg S, Herder C, Walston JD, Zhu Y, Vasan RS, Klopp N, Boerwinkle E, Larson MG, Psaty BM, Peters A, Ballantyne CM, Witteman JC, Hoogeveen RC, Benjamin EJ, Koenig W, Tracy RP. Duffy antigen receptor for chemokines (Darc) polymorphism regulates circulating concentrations of monocyte chemoattractant protein-1 and other inflammatory mediators. Blood. 2009;115:5289–99.
Seki E, de Minicis S, Inokuchi S, Taura K, Miyai K, van Rooijen N, Schwabe RF, Brenner DA. CCR2 promotes hepatic fibrosis in mice. Hepatology. 2009;50:185–97.
Shields PL, Morland CM, Salmon M, Qin S, Hubscher SG, Adams DH. Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C infected livers. J Immunol. 1999;163:6236–43.
Shen HY, Deng YC, Wang QM, Li QS, Xu Z. Expression of MCP-1 in the patients of chronic hepatitis B complicated with nonalcoholic fatty liver disease [Article in Chinese]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2012;28:975–8.
Simpson KJ, Henderson NC, Bone-Larson CL, Lukacs NW, Hogaboam CM, Kunkel SL. Chemokines in the pathogenesis of liver disease: so many players with poor defined roles. Clin Sci. 2003;104:47–63.
Soo HM, Garzino-Demo A, Hong W, Tan YH, Tan YJ, Goh P, Lim SG, Lim SP. Expression of a full-length hepatitis C virus cDNA up-regulates the expression of CC chemokines MCP-1 and RANTES. Virology. 2002;303:253–7.

Szalai C, Duba J, Prohászka Z, Kalina A, Szabó T, Nagy B, Horváth L, Császár A. Involvement of polymorphisms in the chemokine system in the susceptibility for coronary artery disease (CAD). Coincidence of elevated Lp(a) and MCP-1 -2518 G/G genotype in CAD patients. Atherosclerosis. 2001a;158:233–9.

Szalai C, Kozma GT, Nagy A, Bojszkó A, Krikovszky D, Szabó T, Falus A. Polymorphism in the gene regulatory region of MCP-1 is associated with asthma susceptibility and severity. J Allergy Clin Immunol. 2001b;108:375–81.

Thio CL, Astemborski J, Bashirova A, Mosbruger T, Greer S, Witt MD, Goedert JJ, Hilgartner M, Majeske A, O'Brien SJ, Thomas DL, Carrington M. Genetic protection against hepatitis B virus conferred by CCR5Delta32: evidence that CCR5 contributes to viral persistence. J Virol. 2007;81:441–5.

Thursz MR, Kwiatkowski D, Allsopp CE, Greenwood BM, Thomas HC, Hill AV. Association between an MHC class II allele and clearance of hepatitis B virus in the Gambia. N Engl J Med. 1995;332:1065–9.

Traynor TR, Huffnagle GB. Role of chemokines in fungal infection. Med Mycol. 2001;39:41–50.

Traynor TR, Kuziel WA, Toews GB, Huffnagle GB. CCR2 expression determines T1 versus T2 polarization during pulmonary *Cryptococcus neoformans* infection. J Immunol. 2000;164:2021–7.

Traynor TR, Herring AC, Dorf ME, Kuziel WA, Toews GB, Huffnagle GB. Differential roles of CC chemokine ligand/monocyte chemotactic protein-1 and CCR2 2 in the development of T1 immunity. J Immunol. 2002;168:4659–66.

Uchida E, Anan F, Masaki T, Kaneda K, Nawata T, Eshima N, Saikawa T, Yoshimatsu H. Monocyte chemoattractant protein-1 is associated with silent cerebral infarction in patients on haemodialysis. Intern Med J. 2012;42:29–34.

Vincent JL, Gustot T. Sepsis and cirrhosis: many similarities. Acta Gastroenterol Belg. 2010;73:472–8.

Wang WW, Ang SF, Kumar R, Heah C, Utama A, Tania NP, Li H, Tan SH, Poo D, Choo SP, Chow WC, Tan CK, Toh HC. Identification of serum monocyte chemoattractant protein-1 and prolactin as potential tumor markers in hepatocellular carcinoma. PLoS One. 2013;8:e68904.

Woitas RP, Ahlenstiel G, Iwan A, Rockstroh JK, Brackmann HH, Kupfer B, Matz B, Offergeld R, Sauerbruch T, Spengler U. Frequency of the HIV-protective CC chemokine receptor 5-Delta32/Delta32 genotype is increased in hepatitis C. Gastroenterology. 2002;122:1721–8.

Wu L, Paxton WA, Kassam N, Ruffing N, Rottman JB, Sullivan N, Choe H, Sodroski J, Newman W, Koup RA, Mackay CR. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. J Exp Med. 1997;185:1681–91.

Xu ZE, Xie YY, Chen JH, Xing LL, Zhang AH, Li BX, Zhu CM. Monocyte chemotactic protein-1 gene polymorphism and monocyte chemotactic protein-1 expression in Chongqing Han children with tuberculosis [in Chinese]. Zhonghua Er Ke Za Zhi. 2009;47:200–3.

Xue J, Chen F, Wang J, Wu S, Zheng M, Zhu H, Liu Y, He J, Chen Z. Emodin protects against concanavalin A-induced hepatitis in mice through inhibiting activation of the p38 MAPK-NF-kB signaling pathway. Cell Physiol Biochem. 2015;35:1557–70.

Yang B, Houlberg K, Millward A, Demaine A. Polymorphisms of chemokine and chemokine receptor genes in Type 1 diabetes mellitus and its complications. Cytokine. 2004;26:114–21.

Zhou Y, Kurihara T, Ryseck RP, Yang Y, Ryan C, Loy J, Warr G, Bravo R. Impaired macrophage function and enhanced T cell-dependent immune response in mice lacking CCR5, the mouse homologue of the major HIV-1 coreceptor. J Immunol. 1998;160:4018–25.

Hepatic Biomarkers in Diabetes as Modulated by Dietary Phytochemicals

44

Arpita Basu, Paramita Basu, and Timothy J. Lyons

Contents

Abstract

Both type 2 diabetes (T2D) and features of the metabolic syndrome are associated with hepatic insulin resistance, which may gradually progress to nonalcoholic fatty liver disease (NAFLD) and fibrosis. Biomarkers, including those related to

A. Basu (✉)
Department of Nutritional Sciences, 301 Human Sciences, Oklahoma State University, Stillwater, OK, USA
e-mail: arpita.basu@okstate.edu

P. Basu (✉)
Department of Biology, Texas Woman's University, Denton, TX, USA
e-mail: pbasu@twu.edu

T.J. Lyons (✉)
Centre for Experimental Medicine, Queen's University of Belfast, Northern Ireland, UK
e-mail: t.lyons@qub.ac.uk

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_35

glycemia, serum lipid profiles, lipid oxidation, inflammation, and levels of enzymes reflecting hepatocellular damage (e.g., aminotransferases), may reflect liver function in diabetes. New epidemiological data suggest that noninvasive imaging techniques, such as computed tomography (CT) scans, may provide better predictive biomarkers for NAFLD than circulating liver enzymes. Phytochemicals or plant-derived bioactive compounds present in foods, beverages, and herbal supplements have been shown to modulate biomarkers of liver function in clinical trials and mechanistic studies. Mediterranean diet and dietary phytochemicals, such as polyphenols derived from green tea, berries, olive oil, and resveratrol, have been shown to lower liver enzymes, liver fat content, and to improve hepatic insulin resistance and related biomarkers of oxidative stress and inflammation, especially in the presence of adiposity and the metabolic syndrome. Herbs, such as silymarin and those used in traditional Chinese medicine (for example, hawthorn fruit extract), have also been shown to lower hepatic enzymes and markers of oxidative stress and to increase hepatic antioxidant status in patients with NAFLD. Other emerging biomarkers, such as cytokines and microRNAs, are also being evaluated for efficacy in monitoring and predicting liver dysfunction. Thus, selected phytochemicals, especially those occurring naturally in berries and grapes, tea, olives, nuts, and legumes, when used in the context of a low-calorie diet may have a role in treating liver dysfunction. The use of herbal supplements to modulate hepatic biomarkers requires further evaluation of safety and efficacy.

Keywords

Nonalcoholic fatty liver disease • Hepatic insulin resistance • Aminotransferase • Mediterranean diet • Resveratrol • Green tea • Silymarin

List of Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
CBS	Cystathionine β-synthase
CHD	Coronary heart disease
Cox-2	Cyclooxygenase-2
CRP	C-reactive protein
CSE	Cystathionine γ-lyase
CT	Computed tomography
CVD	Cardiovascular disease
GGT	Gamma-glutamyl transferase
H_2S	Hydrogen sulfide
HMG CoA reductase	Hydroxymethylglutaryl-coenzyme A reductase
HOMA-IR	Homeostatic model assessment of insulin resistance
HS	Hepatic steatosis
IL-2R	Interleukin-2 receptor

IL-8	Interleukin 8
Med diet	Mediterranean diet
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
OLETF	Otsuka Long-Evans Tokushima Fatty
PGE2	Prostaglandin E2
PUFA	Polyunsaturated fatty acids
RCT	Randomized controlled trial
SREBP-1c	Sterol regulatory element-binding protein-1c
SREBP-2	Sterol regulatory element-binding protein-2
T2D	Type 2 diabetes
TCM	Traditional Chinese medicine
TGF-α	Transforming growth factor alpha
TPS	Tissue-polypeptide-specific antigen
VLDL	Very low density lipoprotein

Key Facts on Pathophysiology of Diabetes and Liver Disease

- There is a high prevalence of hepatic steatosis in obese adults with diabetes.
- The liver plays an important role in glucose and lipid metabolism and is one of the key target organs for insulin action.
- High levels of blood glucose and free fatty acids and insulin resistance underlie the condition of hepatic steatosis.
- Obesity also contributes to insulin resistance in the liver.
- Fatty liver is associated with increased inflammation and elevated liver enzymes.

Key Facts on Dietary Polyphenols and Liver Function

- Common dietary approaches in fatty liver disease aim to reduce body weight and insulin resistance.
- Polyphenols in green tea, berries, olive oil, and pure resveratrol extracts have been shown to lower liver enzymes (ALT, AST) and liver fat scores and to improve hepatic insulin resistance.
- These polyphenols also lower biomarkers of oxidative stress and inflammation in hepatic steatosis.
- Resveratrol polyphenol extracts decrease fat deposition and cell death in liver.

Key Facts on Mediterranean Diet and Liver Function

- Mediterranean diet is rich in polyphenols, which are present in olive oil, fruits and vegetables, nuts, and legumes.

- Mediterranean diet is also rich in other plant-based compounds such as monounsaturated and omega-3 fatty acids and fiber.
- Mediterranean diet improves features of the metabolic syndrome.
- Mediterranean diet lowers liver fat content and insulin resistance in human studies.
- Compounds in olives have been shown to reduce damage to liver fat and increase antioxidant protection.

Key Facts on Herbal Supplements and Liver Function

- Traditional Chinese medicinal herbs commonly used to treat liver diseases.
- Pooled results from many studies show hawthorn fruit extracts to improve liver and decrease ALT.
- Milk thistle and its active compound silymarin provide antioxidant protection to the liver.
- Human studies show combining silymarin with vitamin E lowers liver enzymes and liver fat.
- Further studies are needed on the safety of these herbs in humans.

Definitions of Words and Terms

ALT	Alanine aminotransferase, a liver enzyme that catalyzes the transfer of an amino group from L-alanine to -α-ketoglutarate and measured as a biomarker of liver health.
AST	Aspartate aminotransferase, a liver enzyme that catalyzes the reversible transfer of an α-amino group between aspartate and glutamate and measured as a biomarker of liver health.
Hepatic steatosis	Abnormal retention of lipids in liver cells.
Insulin resistance	A condition in which the body fails to respond to the action of insulin hormone related to maintaining normal blood glucose levels, especially in insulin-sensitive tissues like liver and muscle.
Mediterranean diet	A diet traditionally followed by Mediterranean countries and is high in olives and olive oil, whole grains, fruits, vegetables, legumes, and fish and low in red meats and processed foods.
Nonalcoholic fatty liver disease	Type of liver disease defined as the presence of $\geq$5% of hepatic fat accumulation but without other liver conditions

	such as hepatitis, alcohol-related liver damage, and liver cancer.
Polyphenols	A class of plant compounds associated with many health benefits; popular foods and beverages high in polyphenols are berry fruits, cocoa, soy, and tea.
Resveratrol	A polyphenol naturally present in peanuts, grapes, red wine, and some berries; has many biological functions that can lower risk factors of chronic diseases such as cardiovascular disease and also improve glucose and lipoid metabolism and liver health.
Silymarin	A natural compound derived from the species *Silybum marianum*, which is commonly known as milk thistle and is associated with promoting liver health.
Type 2 diabetes	A metabolic condition caused by insulin resistance especially in the liver with symptoms such as high blood glucose and triglycerides.

Introduction

The worldwide prevalence of chronic liver disease, and specifically nonalcoholic fatty liver disease (NAFLD), has increased dramatically in recent years (Younossi et al. 2015). The increase in NAFLD parallels that of type 2 diabetes (T2D), and NAFLD further complicates hepatic insulin resistance underlying T2D, emphasizing the need for prevention, and early detection and treatment of these interrelated chronic conditions. NAFLD is now recognized as a hepatic component of the metabolic syndrome, and is defined as the presence of $\geq$5% of hepatic steatosis (HS), in the absence of competing liver disease etiologies, such as chronic viral hepatitis, use of medications that induce steatosis, or significant alcohol consumption (Chalasani et al. 2012). NAFLD may lead to nonalcoholic steatohepatitis (NASH), which is defined histologically by presence of HS with, in addition, evidence of hepatocyte damage and hepatic inflammation, and which may progress to cirrhosis. Early detection and reversal of NAFLD are clearly important goals. Studies have reported clinical and laboratory biomarkers of NAFLD to predict the development of advanced liver fibrosis in NAFLD, especially in context of diabetes. One example is the fatty liver index, which uses triglyceride level and waist circumference to predict NAFLD (Bedogni et al. 2006). Other studies of NAFLD patients have demonstrated that the presence of metabolic syndrome and hypertriglyceridemia, higher aspartate aminotransferase (AST)-to-alanine aminotransferase (ALT) ratio, and lower platelet counts are associated with more advanced liver disease (Loomba et al. 2012). Clinical prediction algorithms have been created to

identify NAFLD patients with and without advanced fibrosis. One example is the well-validated NAFLD fibrosis score, which takes into account age, body mass index (BMI), impaired fasting glucose or diabetes, AST-to-ALT ratio, platelet count, and albumin (Castera et al. 2013). The "FibroScan" index which uses transient elastography to detect liver hardness/elasticity has also become a popular noninvasive biomarker of liver function in recent years (Ding et al. 2015). Furthermore, several serum microRNAs are emerging as more sensitive biomarkers for early detection of liver disease and could complement the conventional biomarkers in monitoring liver dysfunction (Hayes and Chayama 2016). These biomarkers can help to define treatment goals in patients with coexistent T2D and liver disease and involve monitoring indices of glycemia, hepatic insulin resistance, and fat content, as well as serum lipid profiles, according to disease stage and an individual's comorbid conditions. Lifestyle intervention, particularly dietary intervention, is important for all patients, irrespective of disease stage, and dietary factors are the focus of this review. Phytochemicals or plant-based dietary patterns confer protection against many chronic diseases, and emerging epidemiological and clinical trials support their protective role in chronic liver conditions as well. Our objective is to identify and discuss selected dietary interventions involving phytochemical-containing foods, beverages, and herbal supplements in the management of hepatic biomarkers in at-risk populations or patients with clinically evident liver disease.

Type 2 Diabetes and Liver Diseases: Pathophysiology

The prevalence of NAFLD in obese adults with T2D exceeds 70% (Stefan and Häring 2011). The liver plays a critical role in modulating glycemia and insulin sensitivity, and hepatic dysfunction is linked to inflammation, obesity, subclinical cardiovascular disease (CVD), and T2D. The pathogenesis of NAFLD and related hepatic dysfunction is complex and not yet fully understood. Obesity and insulin resistance have been shown to be the major mediators, and the increased flux of free fatty acids from insulin resistant adipocytes to the liver is considered to be the "first hit" leading to HS and hepatic toxicity. NASH has been characterized by apoptosis of liver cells, accelerated by lipotoxic intermediates and diacylglycerol that further worsen insulin resistance in diabetic patients (Petta et al. 2016). Hyperglycemia in prediabetes or overt diabetes provides further substrate for triglyceride synthesis. As triglycerides accumulate in the liver, dysregulation of metabolic pathways leading to oxidation, storage, and secretion of hepatic lipids plays an active role in the pathogenesis of HS. Although hepatic VLDL secretion may be increased four- to fivefold in insulin resistance, contributing to marked hypertriglyceridemia, this is not sufficient to prevent HS (Choi and Ginsberg 2011). Insulin resistance is not only a factor in obesity and diabetes but also may be an underlying mechanism for NAFLD in nonobese, nondiabetic individuals, as noted in a euglycemic insulin clamp study (Bugianesi et al. 2005). In most cases, insulin resistance has been commonly associated with NAFLD in the context of obesity, and the development and

progression of NAFLD has been positively correlated with insulin resistance, as well as a state of positive energy balance due to excess caloric intake and poor dietary quality (Bhatt and Smith 2015). The pathophysiology of NAFLD and HS also involves lipotoxicity, inflammation, oxidative stress, and recently the gut-liver axis interaction (Higuera-de la Tijera and Servín-Caamaño 2015), factors all of which may contribute to insulin resistance. Thus, biomarkers of lipid oxidation and of systemic oxidative stress and inflammation may be monitored to assess treatment responses in chronic liver dysfunction.

Diabetes and NAFLD: Epidemiological Evidence

As summarized in Table 1, several large epidemiological studies have identified strong associations between diabetes and chronic liver diseases. In a meta-analysis, Younossi et al. (2015) estimated the global burden of NAFLD to be 25% of the adult population and the prevalence of diabetes to be 22.5% and 43.6% among NAFLD and NASH patients, respectively. This meta-analysis involved approximately 8.5 million adults, and NAFLD was diagnosed using three different techniques: imaging, liver biopsy, and blood testing. Another significant finding of this meta-analysis was the impact of the diagnostic modality used to detect NAFLD; liver enzyme elevation (blood testing) was found to significantly underestimate the true prevalence of NAFLD compared with imaging techniques in asymptomatic patients without NAFLD at baseline. The high prevalence of metabolic syndrome (43%) and hyperlipidemia (69%) in NAFLD patients suggests that risk stratification is feasible and suggests that monitoring of relevant biomarkers and institution of early interventions may be effective in addressing the burden of CVD in these patients (Younossi et al. 2015). Other retrospective and prospective cohort studies also support the strong positive correlations between NAFLD and the incidence of diabetes (Shah et al. 2015; Wild et al. 2016; Fukuda et al. 2016). These studies support the role of hepatic lipid accumulation ("steatosis"), as assessed by computer tomography, as a weight-independent metabolic biomarker of insulin resistance, subclinical atherosclerosis, and proinflammatory phenotype, factors relevant to both T2D and CVD risk. Both chronic liver disease and T2D have long asymptomatic phases prior to diagnosis, and thus early identification of these conditions based on established and emerging biomarkers has great potential to alleviate their health and economic impacts on society.

Phytochemicals and Hepatic Biomarkers: Dietary Polyphenols

Tables 2 and 3 represent studies in animal models and human clinical trials addressing the efficacy of dietary polyphenols in improving liver function associated with NAFLD. In these studies, dietary polyphenols were derived from green tea, berries, olive oil, and resveratrol, and in most cases lowered liver enzymes (ALT,

Table 1 Epidemiological studies on the associations of diabetes with NAFLD

Author, year	Study design	Subject characteristics	Significant associations
Younossi et al. (2015)	Meta-analysis of 86 studies across the globe (22 countries)	Participants ($N = 8{,}515{,}431$) at risk or with clinical diagnosis of NAFLD	NAFLD correlated with obesity, diabetes, and the metabolic syndrome
Singh et al. (2015)	Case control study	Participants with NAFLD ($N = 461$) vs. controls ($N = 181$)	NAFLD correlated with obesity, diabetes, and the metabolic syndrome
Shah et al. (2015)	Prospective cohort study	Participants from MESA without CVD at baseline ($N = 3153$)	Hepatic steatosis correlated with incidence of T2D
Wild et al. (2016)	Retrospective cohort study	Participants with T2D ($N = 1.8$ million person-years) with data on hospital admissions and deaths due to CLD	High incidence of NAFLD in T2D population
Fukuda et al. (2016)	Retrospective cohort study	Participants with NAFLD with or without overweight ($N = 4629$)	High incidence of T2D in overweight adults with NAFLD
Lomonaco et al. (2016)	Cross-sectional study	Obese participants with T2D and/or NAFLD/NASH versus controls (no T2D or NAFLD) ($N = 154$)	NAFLD associated with severe diabetes and hepatic insulin resistance versus no NAFLD

The above table is a summary of the epidemiological evidence on the associations between diabetes and liver diseases including NAFLD

AST), reduced biomarkers of oxidative stress and inflammation, reduced HS, and improved hepatic insulin resistance. Therapeutic approaches for NAFLD and NASH are often directed at reducing BMI and improving insulin resistance through lifestyle modifications, pharmacological treatments, and recently, bariatric surgery. These studies provide evidence that dietary interventions may also be important and that polyphenols may exert multiple benefits in NAFLD. Polyphenols have been shown to regulate expression of genes involved in *de novo* lipogenesis and fatty acid oxidation, actions that may contribute to their lipid-lowering effects in the liver. Their antioxidant, anti-inflammatory, antifibrogenic, and antilipogenic properties may also inhibit progression of NAFLD. Green tea polyphenols have been shown to protect against liver injury and steatosis in genetic models and dietary fat-induced models of NASH, and studies have shown that green tea extracts reduce hepatic injury in *ob/ob* mice by inhibiting hepatic lipid accumulation and lipid peroxidation, as well as restoring antioxidant defenses and decreasing proinflammatory responses (Park et al. 2012; Chung et al. 2012). Recent studies also demonstrate that resveratrol, a naturally occurring polyphenol in grapes and berries, has beneficial effects in liver disorders mainly mediated by its antioxidant and anti-inflammatory functions (McGill et al. 2015). Resveratrol has been shown to significantly decrease hepatic fat deposition, necrosis, and apoptosis in Wistar rats and provide liver protection against chemical, cholestatic, and alcohol injury (Faghihzadeh et al. 2015a). However, the

Table 2 Preclinical studies on dietary polyphenols and biomarkers of NAFLD

Author, year	Animal model	Polyphenol treatment	Significant hepatic outcomes with polyphenols
Yamabe et al. (2009)	Otsuka Long-Evans Tokushima Fatty (OLETF) rats (model of type 2 diabetes)	Matcha green tea (50, 100, or 200 mg/kg/day); orally for 16 weeks	Decrease in hepatic lipids and increase in SREBP-2 expression
Chung et al. (2012)	Obese mice (ob/ob) and their 5-weeks-old C57BL6 lean littermates	GTE (0%, 0.5% or 1%); orally for 6 weeks	Decrease in hepatic steatosis, fat oxidation, inflammation, and ALT
Xu et al. (2015)	ApoE(−/−) mice fed a western-type diet	Apple polyphenols (100 mg/kg) or atorvastatin (10 mg/kg) for 12 weeks	Decrease in hepatic steatosis and oxidative stress
Chung et al. (2015)	Male Wistar rats fed a low or high fat diet	GTE (0%, 1% or 2%); orally for 8 weeks	Decrease in hepatic steatosis and inflammation; decrease in hepatic COX-2 and PGE2
de Oliveira et al. (2015)	C57BL/6 mice fed 10% or 60% fat	Acai seed extract, 300 mg/kg body weight/day for 12 weeks	Decreased hepatic steatosis, oxidative stress, and SREBP-1c and HMG CoA reductase expressions
Lepore et al. (2015)	Male C57BL/6JOlaHsd mice fed a standard or cafeteria diet	Oleuropein (Ole) (0.037 mmol/kg/day) and acetylated ole (0.025 mmol/kg/day) for 15 weeks	Decrease in hepatic steatosis and insulin resistance induced by the cafeteria diet

The above table is a summary of the effects of dietary polyphenols on hepatic biomarkers in animal models of diabetes and NAFLD

results have been somewhat conflicting in reported human studies examining the effects of resveratrol in adult patients with NAFLD.

Over 12 weeks, resveratrol supplementation (500 or 600 mg/day) significantly improved liver function, decreasing liver fat content and inflammation, in overweight participants with NAFLD (Faghihzadeh et al. 2014, 2015b; Chen et al. 2015). On the other hand, in an 8-week study, a larger dose of resveratrol (3000 mg/day) did not improve features of NAFLD in overweight or obese adults but increased liver enzymes suggesting hepatic stress in response to large doses of the supplement (Chachay et al. 2014). Thus, based on findings from preclinical studies, dietary polyphenols may effectively ameliorate the pathological reactions underlying both T2D and chronic liver diseases and therefore should be considered in the primary and secondary prevention of these diseases. Larger clinical trials on the effects of dietary polyphenols (single or in combination; low vs. high dose) on biomarkers of liver function and

Table 3 Clinical trials on dietary polyphenols and biomarkers of NAFLD

Author, year	Study design	Subject characteristics	Polyphenol treatment	Significant hepatic outcomes with polyphenols
Sakata et al. (2013)	RCT, 12 weeks	Participants with features of NAFLD ($N = 17$); age: 51 ± 9y BMI: 29 ± 3 kg/m^2	Green tea (high and low dose catechins) versus placebo	Decrease in ALT and hepatic fat content
Chang et al. (2014)	RCT, 12 weeks	Participants with overweight/obesity and fatty liver ($N = 36$); age: 38 ± 9y BMI: 31 ± 4 kg/m^2	HSE capsules (2700 mg) versus placebo	Improved liver steatosis; decreased liver fat scores
Guo et al. (2014)	RCT, 4 weeks	Participants with features of NAFLD ($N = 44$); age: 21 ± 1y BMI: ≥23 kg/m^2	Bayberry juice (0.5 L; 1350 mg polyphenols) or placebo	Decreased NAFLD-related biomarkers of oxidative stress, inflammation, and apoptosis (protein carbonyls, IL-8, TPS)
Faghihzadeh et al. (2014)	RCT, 12 weeks	Participants with features of NAFLD ($N = 50$); age: 45 ± 9y BMI: 28 ± 3 kg/m^2	Resveratrol capsules (500 mg) versus placebo	Decrease in ALT, inflammatory cytokines, nuclear factor *k*B activity, serum cytokeratin-18, and hepatic steatosis
Faghihzadeh et al. (2015b)	RCT, 12 weeks	Participants with features of NAFLD ($N = 50$); age: 45 ± 9y BMI: 28 ± 3 kg/m^2	Resveratrol capsules (500 mg) versus placebo	Decrease in ALT and hepatic steatosis
Chen et al. (2015)	RCT, 12 weeks	Participants with features of NAFLD ($N = 60$); age: 44 ± 10y BMI: 26 ± 3 kg/m^2	Resveratrol capsules (600 mg) versus placebo	Decrease in ALT and AST and inflammatory biomarkers

The above table is a summary of the effects of dietary polyphenols on hepatic biomarkers in patients with NAFLD

diabetes are needed to support or refute their use in the prevention and management of these interdependent metabolic conditions and to define optimal doses.

Phytochemicals and Hepatic Biomarkers: Plant-Based Diets

As summarized in Table 4, plant-based diets, the Mediterranean diet, and diets that have high content of olive oil have been associated with improved liver function in clinical trials ranging from 6 weeks to 6 months. Given the strong link between

Table 4 Clinical trials on plant-based dietary interventions with or without nutraceuticals and biomarkers of NAFLD

Author, year	Study design	Subject characteristics	Dietary treatment	Significant hepatic outcomes with diet and/or nutraceuticals
Sofi et al. (2010)	RCT, 12 months	Participants with features of NAFLD ($N = 11$); age: 55 (30–70y) BMI: 29 ± 4 kg/m^2	Olive oil with n-3 PUFA (6.5 mL/day) versus control	Decrease in liver enzymes (AST, ALT, GGT) and fat scores
Ryan et al. (2013)	RCT, crossover 6 weeks	Participants with features of NAFLD ($N = 12$); age: 55 ± 14y BMI: 32 ± 4 kg/m^2	Med diet, low fat high carbohydrate diet, or control diet	Decrease in hepatic steatosis and insulin resistance after Med diet
Kani et al. (2014)	RCT, crossover 8 weeks	Participants with features of NAFLD ($N = 45$); age: 47 ± 3y BMI: 30 ± 3 kg/m^2	Low-calorie diet; low-calorie, low-carbohydrate diet; or low-calorie, low-carbohydrate soy-containing diet	Decrease in ALT and AST, and fibrinogen after the soy diet
Nigam et al. (2014)	RCT, 6 months	Participants with features of NAFLD ($N = 93$); age: 37 ± 6y BMI: 27 ± 4 kg/m^2	Olive oil, canola oil, or soybean/safflower oil (control) as cooking medium (not exceeding 20 g/day) along with counseling for therapeutic lifestyle changes	Improvements in grading of fatty liver, liver span, insulin resistance, and lipids with use of canola and olive oil
Abenavoli et al. (2015)	RCT, 6 months	Participants with features of NAFLD ($N = 30$); age: 50 ± 9y BMI: 28 ± 3 kg/m^2	Med diet, Med diet + Realsil complex (silybin-vitamin E-phospholipids) vs. control	No significant effects on hepatic biomarkers; decrease in HOMA-IR
Trovato et al. (2015)	Pre- and postintervention, 6 months	Participants with features of NAFLD ($N = 90$); age: 50 ± 14y BMI: 31 ± 5 kg/m^2	Counseling to increase adherence to the Med diet	Decrease in liver fat content based on bright liver score

The above table is a summary of the effects of plant-based dietary interventions with or without nutraceuticals on biomarkers of NAFLD

NAFLD, insulin resistance, and the metabolic syndrome, and a lack of specific dietary guidelines for NAFLD, current dietary approaches are primarily focused on CVD risk factors. The accumulated data obtained so far suggest that westernized dietary patterns, characterized by a high consumption of red meat products, refined grains, pastries, and sugar-sweetened beverages, are associated with higher likelihood or risk of the metabolic syndrome, whereas adherence to diets that are rich in whole grains, fruits, vegetables, legumes, and fish appears to have beneficial effects (Andersen and Fernandez 2013). Among prudent dietary patterns, the Mediterranean diet has been given special attention because evidence obtained from epidemiological studies and clinical trials supports its beneficial effect on both the prevention and the resolution of the metabolic syndrome (Esposito et al. 2013). In a recently reported study of 642 patients with coronary heart disease (CHD) randomized to the Mediterranean diet (35% fat; 22% from monounsaturated fatty acids) versus a low-fat diet (<28% fat), significant findings related to liver function were revealed. Although both diets improved insulin sensitivity, the low-fat diet had the greatest benefit for patients with hepatic insulin resistance, whereas the Mediterranean diet was more beneficial for those with muscle insulin resistance or combined muscle and liver insulin resistance (Blanco-Rojo et al. 2016). While many foods and beverages within the Mediterranean diet may act synergistically, olive oil in particular has been shown to exert protective effects against hepatic oxidative stress in animal models. In mice with high fat diet-induced hepatic stress, supplementation with tyrosol, a major polyphenol in olive oil, significantly increased hepatic cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) expression, as well as hydrogen sulfide (H_2S) synthesis. Such effects were associated with the attenuation of high fat diet-induced hepatic lipid peroxidation and with restoration of the redox equilibrium via the antioxidant glutathione (Sarna et al. 2015). These findings further strengthen the hepatoprotective effects of the Mediterranean diet and warrant further investigation in populations habituated to western high-fat diets.

Phytochemicals and Hepatic Biomarkers: Functional Foods and Herbal Supplements

Phytochemical-rich functional foods and herbal supplements, including those used in traditional Chinese medicine (TCM), have demonstrated beneficial effects against liver damage, metabolic syndrome, and T2D. As summarized in Table 5, specific functional foods, such as berries, oats, and olive oil, as well as herbs such as silymarin and hawthorn fruit extracts significantly improved liver function both in healthy adults and those with NAFLD. Functional foods, such as green tea, berries, olive oil, and oats are rich in polyphenolic flavonoids and in many other nutrients and bioactive compounds such as vitamins, soluble fiber, and monounsaturated fatty acids that have been consistently shown to alleviate cardiometabolic risks and liver dysfunction in clinical and mechanistic studies (Valtueña et al. 2008; López-Miranda et al. 2010; Liu et al. 2013; Chang et al. 2013). TCM has been used to treat liver disease since ancient times. The *Yellow Emperor's Internal Classic*, an old scripture

Table 5 Clinical trials on functional foods and herbal treatment and biomarkers of NAFLD

Author, year	Study design	Subject characteristics	Polyphenol treatment	Significant hepatic outcomes with foods/herbal therapy
Valtueña et al. (2008)	RCT, crossover 2 weeks	Healthy adults ($N = 33$); age: 61 ± 4y BMI: 27 ± 3 kg/m^2	High versus low total antioxidant capacity foods and beverages included in diet (e.g., berries, red wine, olive oil)	Decreased ALT, AST, and GGT after high antioxidant capacity diet
Shi et al. (2012)	Meta-analysis of 62 RCTs involving 20 herbs used in TCM	Patients with NAFLD ($N = 5{,}904$) on treatment	TCM versus conventional treatment; hawthorn fruit most commonly used	Decrease in ALT and liver steatosis after TCM treatment
Hajiaghamohammadi et al. (2012)	RCT, 8 weeks	Participants with features of NAFLD (N = 66); age: 33 ± 6y BMI: 27 ± 2 kg/m^2	Metformin, pioglitazone, or silymarin	Largest reduction in ALT and AST in silymarin group
Chang et al. (2013)	RCT, 12 weeks	Participants with features of NAFLD ($N = 34$); age: 18–65y BMI: ≥27 kg/m^2	Oat cereal or placebo	Decrease in ALT and abdominal adiposity
Sorrentino et al. (2015)	RCT, 12 weeks	Participants with features of NAFLD ($N = 78$); age: 56 ± 13y BMI: 31 ± 5 kg/m^2	Eurosil 85(®)-based nutraceutical (silymarin + vitamin E) versus controls in presence of Med diet + brisk walking	Decrease in liver steatosis based on decreased hepatic steatosis index and lipid accumulation product with silymarin + vitamin E
Aller et al. (2015)	RCT, 12 weeks	Participants with features of NAFLD ($N = 36$); age: 47 ± 11y BMI: 36 ± 4 kg/m^2	Eurosil 85(®)-based nutraceutical (silymarin + vitamin E) versus controls in presence of hypocaloric diet + exercise	Decreased liver enzymes and fat scores with silymarin + vitamin E

The above table is a summary of the effects of selected functional foods and herbal therapy on hepatic biomarkers of NAFLD

bearing records of TCM, shows that TCM has been used for liver diseases in China at least since 475 BC. In the meta-analysis of 62 randomized controlled trials (RCTs) reported by Shi et al. (2012), certain TCM herbs were shown to significantly improve liver function (decrease ALT) when compared to conventional western medicine treatment (Shi et al. 2012): hawthorn fruit, danshen root, and oriental water plantain rhizome were the top three most frequently used TCM herbs in these trials. Herbal supplements, such as silymarin, have also been widely used in the treatment of liver disorders. Silymarin is the bioactive compound contained in the medicinal plant "milk thistle," native to the Mediterranean region, and traditionally has been associated with hepatoprotective effects. Silymarin has been shown to promote regeneration of liver cells, increase antioxidant factors such as glutathione in liver cells, and also reduce collagen accumulation and risks of hepatic fibrosis (Vargas-Mendoza et al. 2014). Reported human studies have demonstrated the hepatoprotective effects of silymarin alone, as well in combination with healthy diets, such as the Mediterranean diet, or other antioxidant micronutrients in improving insulin resistance and liver function. In a clinical study of patients with NASH, silymarin supplementation for 8 weeks was associated with a significant decrease in hepatic enzymes when compared to the control group (Solhi et al. 2014). In a 6-month study, overweight patients with NAFLD randomized to the Mediterranean diet alone or in combination with supplements containing silymarin showed improvements in insulin resistance and plasma lipids compared to the control group (Abenavoli et al. 2015). Silymarin in combination with vitamin E has also been shown to be protective against hepatic oxidative stress and in decreasing liver enzymes in overweight and obese adults with NAFLD (Hajiaghamohammadi et al. 2012; Sorrentino et al. 2015; Aller et al. 2015). While the evidence based on reported literature is supportive and shows efficacy in liver diseases, further studies are warranted on the safety of herbal supplements and their modulation of hepatic biomarkers in high risk patients.

Potential Applications to Prognosis, Other Diseases, or Conditions

Several biomarkers of the metabolic syndrome, diabetes, and NAFLD are typically used in assessing the presence of risk factors and the progression of liver dysfunction. Liver disease is typically defined as $\geq$ twofold elevation of AST or ALT and has also been associated with biomarkers of autoimmunity and cardiometabolic alterations (Liu et al. 2015). Based on epidemiological studies, circulating liver enzymes alone have less prognostic value when compared to imaging techniques, such as computed tomography and liver histology in monitoring liver disease progression (Younossi et al. 2015). However, liver enzymes, especially ALT, continue to be used widely as biomarkers of liver function in combination with other markers, such as selected cytokines [interleukin (IL)-2 receptor (R) and transforming growth factor alpha (TGF-α)], and AST:platelet ratio index in predicting liver fibrosis (Deng et al. 2015). Serum microRNAs (miR), a class of

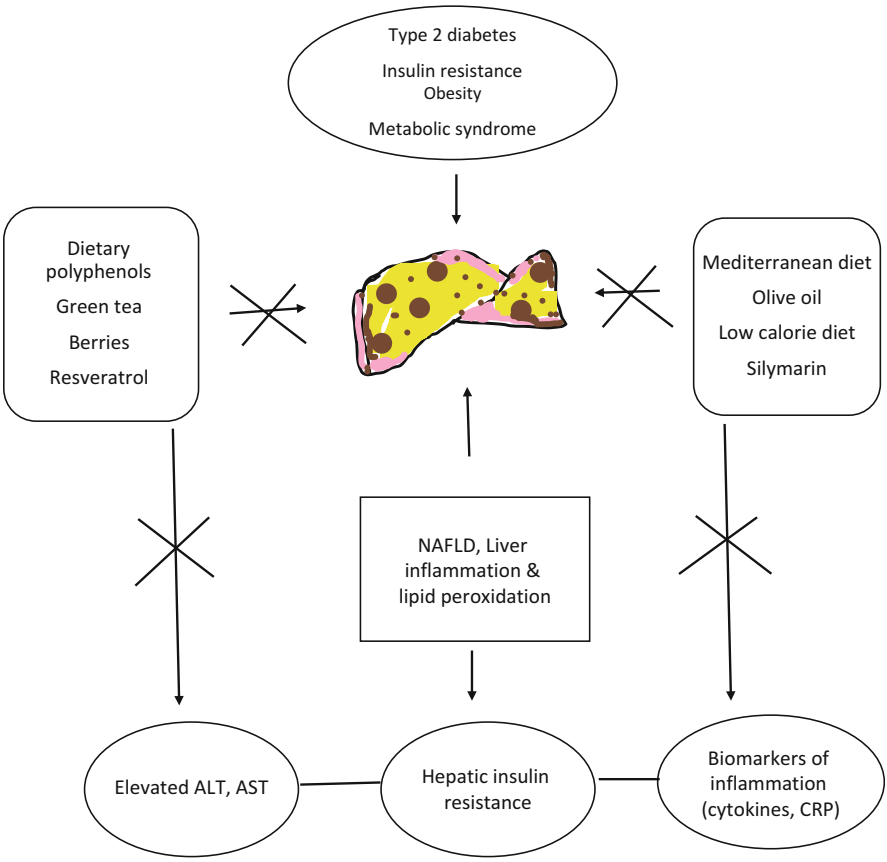

Fig. 1 Hepatic biomarkers, risk factors, and phytochemicals. The above figure is a summary of the risk factors associated with liver dysfunction in diabetes and the modulation of biomarkers by phytochemical-rich diet, functional foods and beverages, and herbs. *Cross sign* indicates inhibition or downregulation

small noncoding RNAs, have also been shown to play an important role in regulating gene expression associated with liver function. Liver enzymes, such as AST and alkaline phosphatase, though widely used in assessing liver function are also expressed in muscle and bone tissues, and this somewhat limits their specificity and clinical interpretation. In contrast miRs have greater specificity, e.g., miR-122 is specific to the liver, representing 70% of the liver miRNA, and correlates strongly with liver enzyme levels, necroinflammatory activity, and the degree of fibrosis. Several miRNAs, such as miR-21 and miR-181b, promote liver fibrosis, while miR-29b and miR-122 prevent liver fibrosis and lower inflammation (Hayes and Chayama 2016). These emerging biomarkers must be evaluated in future intervention studies, including those assessing plant-based diets and bioactive compounds to examine their effectiveness in predicting disease progression and liver function when compared to classical disease biomarkers (Fig. 1).

Summary Points

- Obesity, metabolic syndrome, and T2D are strong risk factors of chronic liver conditions, especially NAFLD and HS.
- Insulin resistance underlies the pathophysiology connecting diabetes with liver dysfunction.
- Liver enzymes, such as ALT and AST, are widely used biomarkers of liver function.
- Mediterranean diet improves liver function and coexistent metabolic syndrome and T2D.
- Polyphenol containing foods and beverages lower biomarkers of lipid oxidation and inflammation in liver dysfunction.
- Herbal supplements such as silymarin promote liver function but need further evaluation regarding safety and effects on selected biomarkers of liver diseases.

References

Abenavoli L, Greco M, Nazionale I, et al. Effects of Mediterranean diet supplemented with silybin-vitamin E-phospholipid complex in overweight patients with non-alcoholic fatty liver disease. Expert Rev Gastroenterol Hepatol. 2015;9:519–27.

Aller R, Izaola O, Gómez S, et al. Effect of silymarin plus vitamin E in patients with non-alcoholic fatty liver disease. A randomized clinical pilot study. Eur Rev Med Pharmacol Sci. 2015;19:3118–24.

Andersen CJ, Fernandez ML. Dietary strategies to reduce metabolic syndrome. Rev Endocr Metab Disord. 2013;14:241–54.

Bedogni G, Bellentani S, Miglioli L, et al. The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. BMC Gastroenterol. 2006;6:33.

Bhatt HB, Smith RJ. Fatty liver disease in diabetes mellitus. Hepatobiliary Surg Nutr. 2015;4:101–8.

Blanco-Rojo R, Alcala-Diaz JF, Wopereis S, et al. The insulin resistance phenotype (muscle or liver) interacts with the type of diet to determine changes in disposition index after 2 years of intervention: the CORDIOPREV-DIAB randomised clinical trial. Diabetologia. 2016; 59:67–76.

Bugianesi E, Gastaldelli A, Vanni E, et al. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. Diabetologia. 2005;48:634–42.

Castera L, Vilgrain V, Angulo P. Noninvasive evaluation of NAFLD. Nat Rev Gastroenterol Hepatol. 2013;10:666–75.

Chachay VS, Macdonald GA, Martin JH, et al. Resveratrol does not benefit patients with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol. 2014;12:2092–103.e1–6.

Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. Hepatology. 2012;55:2005–23.

Chang HC, Huang CN, Yeh DM, et al. Oat prevents obesity and abdominal fat distribution, and improves liver function in humans. Plant Foods Hum Nutr. 2013;68:18–23.

Chang HC, Peng CH, Yeh DM, Kao ES, Wang CJ. *Hibiscus sabdariffa* extract inhibits obesity and fat accumulation, and improves liver steatosis in humans. Food Funct. 2014;5:734–9.

Chen S, Zhao X, Ran L, et al. Resveratrol improves insulin resistance, glucose and lipid metabolism in patients with non-alcoholic fatty liver disease: a randomized controlled trial. Dig Liver Dis. 2015;47:226–32.

Choi SH, Ginsberg HN. Increased very low density lipoprotein (VLDL) secretion, hepatic steatosis, and insulin resistance. Trends Endocrinol Metab. 2011;22:353–63.

Chung MY, Park HJ, Manautou JE, Koo SI, Bruno RS. Green tea extract protects against nonalcoholic steatohepatitis in ob/ob mice by decreasing oxidative and nitrative stress responses induced by proinflammatory enzymes. J Nutr Biochem. 2012;23:361–7.

Chung MY, Mah E, Masterjohn C, et al. Green tea lowers hepatic COX-2 and prostaglandin E2 in rats with dietary fat-induced nonalcoholic steatohepatitis. J Med Food. 2015;18:648–55.

de Oliveira PR, da Costa CA, de Bem GF. Euterpe oleracea Mart.-derived polyphenols protect mice from diet-induced obesity and fatty liver by regulating hepatic lipogenesis and cholesterol excretion. PLoS One. 2015;10:e0143721.

Deng YQ, Zhao H, Ma AL, China HepB Related Fibrosis Assessment Research Group, et al. Selected cytokines serve as potential biomarkers for predicting liver inflammation and fibrosis in chronic hepatitis B patients with normal to mildly elevated aminotransferases. Medicine (Baltimore). 2015;94:e2003.

Ding D, Li H, Liu P, et al. FibroScan, aspartate aminotransferase and alanine aminotransferase ratio (AAR), aspartate aminotransferase to platelet ratio index (APRI), fibrosis index based on the 4 factor (FIB-4), and their combinations in the assessment of liver fibrosis in patients with hepatitis B. Int J Clin Exp Med. 2015;8:20876–82.

Esposito K, Kastorini CM, Panagiotakos DB, Giugliano D. Mediterranean diet and metabolic syndrome: an updated systematic review. Rev Endocr Metab Disord. 2013;14:255–63.

Faghihzadeh F, Adibi P, Rafiei R, Hekmatdoost A. Resveratrol supplementation improves inflammatory biomarkers in patients with nonalcoholic fatty liver disease. Nutr Res. 2014;34:837–43.

Faghihzadeh F, Hekmatdoost A, Adibi P. Resveratrol and liver: a systematic review. J Res Med Sci. 2015a;20:797–810.

Faghihzadeh F, Adibi P, Hekmatdoost A. The effects of resveratrol supplementation on cardiovascular risk factors in patients with non-alcoholic fatty liver disease: a randomised, double-blind, placebo-controlled study. Br J Nutr. 2015b;114:796–803.

Fukuda T, Hamaguchi M, Kojima T, et al. The impact of non-alcoholic fatty liver disease on incident type 2 diabetes mellitus in non-overweight individuals. Liver Int. 2016;36:275–83.

Guo H, Zhong R, Liu Y, et al. Effects of bayberry juice on inflammatory and apoptotic markers in young adults with features of non-alcoholic fatty liver disease. Nutrition. 2014;30:198–203.

Hajiaghamohammadi AA, Ziaee A, Oveisi S, Masroor H. Effects of metformin, pioglitazone, and silymarin treatment on non-alcoholic Fatty liver disease: a randomized controlled pilot study. Hepat Mon. 2012;12:e6099.

Hayes CN, Chayama K. MicroRNAs as biomarkers for liver disease and hepatocellular carcinoma. Int J Mol Sci. 2016;17:280.

Higuera-de la Tijera F, Servín-Caamaño AI. Pathophysiological mechanisms involved in non-alcoholic steatohepatitis and novel potential therapeutic targets. World J Hepatol. 2015;7:1297–301.

Kani AH, Alavian SM, Esmaillzadeh A, Adibi P, Azadbakht L. Effects of a novel therapeutic diet on liver enzymes and coagulating factors in patients with non-alcoholic fatty liver disease: a parallel randomized trial. Nutrition. 2014;30:814–21.

Lepore SM, Morittu VM, Celano M, et al. Oral administration of Oleuropein and its semisynthetic peracetylated derivative prevents hepatic steatosis, hyperinsulinemia, and weight gain in mice fed with high fat cafeteria diet. Int J Endocrinol. 2015;2015:431453.

Liu K, Zhou R, Wang B, et al. Effect of green tea on glucose control and insulin sensitivity: a meta-analysis of 17 randomized controlled trials. Am J Clin Nutr. 2013;98:340–8.

Liu Y, Yu J, Oaks Z, et al. Liver injury correlates with biomarkers of autoimmunity and disease activity and represents an organ system involvement in patients with systemic lupus erythematosus. Clin Immunol. 2015;160:319–27.

Lomonaco R, Bril F, Portillo-Sanchez P, et al. Metabolic impact of nonalcoholic steatohepatitis in obese patients with type 2 diabetes. Diabetes Care. 2016;39:632; pii: dc151876. [Epub ahead of print].

Loomba R, Abraham M, Unalp A, Nonalcoholic Steatohepatitis Clinical Research Network, et al. Association between diabetes, family history of diabetes, and risk of nonalcoholic steatohepatitis and fibrosis. Hepatology. 2012;56:943–51.

López-Miranda J, Pérez-Jiménez F, Ros E, et al. Olive oil and health: summary of the II international conference on olive oil and health consensus report, Jaén and Córdoba (Spain) 2008. Nutr Metab Cardiovasc Dis. 2010;20:284–94.

McGill MR, Du K, Weemhoff JL, Jaeschke H. Critical review of resveratrol in xenobiotic-induced hepatotoxicity. Food Chem Toxicol. 2015;86:309–18.

Nigam P, Bhatt S, Misra A, et al. Effect of a 6-month intervention with cooking oils containing a high concentration of monounsaturated fatty acids (olive and canola oils) compared with control oil in male Asian Indians with nonalcoholic fatty liver disease. Diabetes Technol Ther. 2014;16:255–61.

Park HJ, Lee JY, Chung MY, et al. Green tea extract suppresses NFkB activation and inflammatory responses in diet-induced obese rats with nonalcoholic steatohepatitis. J Nutr. 2012;142:57–63.

Petta S, Valenti L, Bugianesi E, Special Interest Group on Personalised Hepatology of the Italian Association for the Study of the Liver (AISF), Special Interest Group on Personalised Hepatology of the Italian Association for the Study of the Liver AISF, et al. A "systems medicine" approach to the study of non-alcoholic fatty liver disease. Dig Liver Dis. 2016;48:333–42.

Ryan MC, Itsiopoulos C, Thodis T, et al. The Mediterranean diet improves hepatic steatosis and insulin sensitivity in individuals with non-alcoholic fatty liver disease. J Hepatol. 2013;59:138–43.

Sakata R, Nakamura T, Torimura T, Ueno T, Sata M. Green tea with high-density catechins improves liver function and fat infiltration in non-alcoholic fatty liver disease (NAFLD) patients: a double-blind placebo-controlled study. Int J Mol Med. 2013;32:989–94.

Sarna LK, Sid V, Wang P, Siow YL, House JD, Kamin O. Tyrosol attenuates high fat diet-induced hepatic oxidative stress: potential involvement of cystathionine β-synthase and cystathionine γ-lyase. Lipids. 2015;51:583. [Epub ahead of print].

Shah RV, Allison MA, Lima JA, et al. Liver fat, statin use, and incident diabetes: the Multi-Ethnic Study of Atherosclerosis. Atherosclerosis. 2015;242:211–7.

Shi KQ, Fan YC, Liu WY, et al. Traditional Chinese medicines benefit to nonalcoholic fatty liver disease: a systematic review and meta-analysis. Mol Biol Rep. 2012;39:9715–22.

Singh SP, Singh A, Misra D, et al. Risk factors associated with non-alcoholic fatty liver disease in Indians: a case–control study. J Clin Exp Hepatol. 2015;5:295–302.

Sofi F, Giangrandi I, Cesari F, et al. Effects of a 1-year dietary intervention with n-3 polyunsaturated fatty acid-enriched olive oil on non-alcoholic fatty liver disease patients: a preliminary study. Int J Food Sci Nutr. 2010;61:792–802.

Solhi H, Ghahremani R, Kazemifar AM, Hoseini Yazdi Z. Silymarin in treatment of non-alcoholic steatohepatitis: a randomized clinical trial. Caspian J Intern Med. 2014;5:9–12.

Sorrentino G, Crispino P, Coppola D, De Stefano G. Efficacy of lifestyle changes in subjects with non-alcoholic liver steatosis and metabolic syndrome may be improved with an antioxidant nutraceutical: a controlled clinical study. Drugs R&D. 2015;15:21–5.

Stefan N, Häring HU. The metabolically benign and malignant fatty liver. Diabetes. 2011;60:2011–7.

Trovato FM, Catalano D, Martines GF, Pace P, Trovato GM. Mediterranean diet and non-alcoholic fatty liver disease: the need of extended and comprehensive interventions. Clin Nutr. 2015;34:86–8.

Valtueña S, Pellegrini N, Franzini L, et al. Food selection based on total antioxidant capacity can modify antioxidant intake, systemic inflammation, and liver function without altering markers of oxidative stress. Am J Clin Nutr. 2008;87:1290–7.

Vargas-Mendoza N, Madrigal-Santillán E, Morales-González A, et al. Hepatoprotective effect of silymarin. World J Hepatol. 2014;6:144–9.

Wild SH, Morling JR, McAllister DA, Scottish and Southampton Diabetes and Liver Disease Group and the Scottish Diabetes Research Network Epidemiology Group, et al. Type 2 diabetes, chronic liver disease and hepatocellular cancer: a national retrospective cohort study using linked routine data. J Hepatol. 2016. doi:10.1016/j.jhep.2016.01.014. pii: S0168-8278(16) 00020-9. [Epub ahead of print].

Xu ZR, Li JY, Dong XW, et al. Apple polyphenols decrease atherosclerosis and hepatic steatosis in ApoE−/− mice through the ROS/MAPK/NF-kB pathway. Nutrients. 2015;7:7085–105.

Yamabe N, Kang KS, Hur JM, Yokozawa T. Matcha, a powdered green tea, ameliorates the progression of renal and hepatic damage in type 2 diabetic OLETF rats. J Med Food. 2009;12:714–21.

Younossi ZM, Koenig AB, Abdelatif D, et al. Global epidemiology of non-alcoholic fatty liver disease-meta-analytic assessment of prevalence, incidence and outcomes. Hepatology. 2015. doi:10.1002/hep.28431. [Epub ahead of print].

Inflammatory Biomarkers in Ascites

45

Philipp Lutz, Hans Dieter Nischalke, and Ulrich Spengler

Contents

Abstract

Ascites formation is the most frequent complication of liver cirrhosis. It offers the unique opportunity to gain samples from body fluid other than blood. In clinical

P. Lutz (✉) • H.D. Nischalke (✉) • U. Spengler (✉)
Department of Internal Medicine I, University of Bonn, Bonn, Germany
e-mail: philipp.lutz@ukb.uni-bonn.de; hans_dieter.nischalke@ukb.uni-bonn.de; ulrich.spengler@ukb.uni-bonn.de

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_3

practice, however, analysis of ascites is limited to total protein to assess the risk for spontaneous bacterial peritonitis (SBP) and to PMN counts to establish the diagnosis of SBP, respectively. By definition, diagnosis of SBP is made if ascites PMN count exceeds 250 cells/mm^3. Research for alternative biomarkers for SBP is ongoing. Urinary test sticks calibrated to ascites, lactoferrin and calprotectin have shown promising results. In addition, ascites calprotectin may confer prognostic information. Several inflammatory cytokines are increased in ascites during episodes of SBP. The presence of bacterial DNA in the ascites has been linked to bacterial translocation but lacks sensitivity and specificity for use as clinical biomarker.

Keywords

Ascites • Spontaneous bacterial peritonitis • Calprotectin • PMN • Liver • Cirrhosis • Ascites total protein

List of Abbreviations

AUROC	Area under the receiver operator curve
CEA	Carcinoembryogenic antigen
CT	Computed tomography
ELISA	Enzyme-linked immunosorbent assay
NGAL	Neutrophil gelatinase-associated lipocalin
PMN	Polymorphonuclear
SAAG	Serum-ascites albumin gradient
SBP	Spontaneous bacterial peritonitis
TNF-alpha	Tumor necrosis factor-alpha

Key Facts of Spontaneous Bacterial Peritonitis

– Spontaneous bacterial peritonitis (SBP) is a particular form of peritonitis without any clinically evident source of infection. It is classified as primary peritonitis which occurs in patients with liver cirrhosis and ascites. In most cases, the suspected route of infection is attributed to bacterial translocation from the intestine, but any kind of bacteremia may result in SBP. Diagnosis of SBP is made if neutrophil counts in the ascites exceed 250 cells/mm^3 in the absence of a contiguous source of infection, e.g., bowel perforation or an intra-abdominal abscess. Diagnosis cannot be made by clinical examination or blood tests. Detection of bacteria by microbiological culture of ascites fluid is achieved only in 50–80% of patients. Treatment includes antibiotics and albumin given intravenously to prevent acute kidney failure. Mortality of SBP is high, ranging from 20% to 40%. Patients who survive a first episode of SBP and patients without SBP who are considered to be at a particular high risk for severe SBP should receive antibiotic prophylaxis to prevent SBP.

Definitions of Words and Terms

- Ascites is peritoneal fluid that often accumulates in patients with liver disease so that removal of ascites by paracentesis becomes necessary for diagnostic or therapeutic reasons.
- Spontaneous bacterial peritonitis is a primary form of peritonitis occurring in patients with liver cirrhosis and ascites by bacterial translocation to the ascites. Diagnosis is made if ascites neutrophils are elevated.
- Bacterascites describes ascites samples in which bacterial growth is detected by conventional culture, but neutrophils in the ascites are below the diagnostic threshold of spontaneous bacterial peritonitis.

Introduction

Ascites, the accumulation of fluid in the peritoneal cavity, occurs under different pathological conditions (Table 1). Therefore, the presence of ascites should not be mistaken as unequivocal sign of advanced liver disease. Assessment of the serum-ascites albumin gradient (SAAG) may help to differentiate between different etiologies causing ascites: a SAAG $\geq$11 g/L indicates that ascites formation is due to chronic liver disease (European Association for the Study of the Liver 2010). This chapter will focus on ascites due to liver disease. It should be kept in mind that the contents of this text may not be applicable to ascites of other origin. All biomarkers summarized in this chapter are listed in Table 2.

If ascites occurs in liver disease, it reflects severely compromised liver function and poor prognosis. The amount of ascites is often considerable, so that peritoneal fluid can safely be aspirated by paracentesis. Thus, ascites enables direct access to a body fluid other than blood. Consequently, aspiration of ascites should offer a unique opportunity to analyze biomarkers in order to assess the degree of liver disease and its complications. However, only parameters to diagnose spontaneous bacterial peritonitis (SBP) and to evaluate the future risk for SBP have been established in clinical practice. SBP is a frequent infectious complication of patients with liver cirrhosis and ascites associated with a high mortality (Wiest et al. 2012; Lutz et al. 2015a). It is a distinct form of primary peritonitis, which occurs without any evident source of infection. SBP is supposed to result from bacterial translocation from the intestine and from bacteremia. Studies on biomarkers in the ascites as diagnostic and prognostic markers of other conditions related to chronic liver disease are scarce, but research on ascites biomarkers is ongoing. The levels of biomarkers in ascites may be influenced by many confounding factors. Table 3 lists some factors that should be considered when assessing new biomarkers. After a short section on the methods used to measure ascites biomarkers, this chapter will focus on established ascites biomarkers which are used to diagnose SBP, to assess the risk to acquire SBP, and to distinguish SBP from secondary peritonitis and tuberculous peritonitis. Finally, ascites biomarkers that have been investigated, but have not been introduced into clinical practice, will be discussed.

Table 1 Common causes of ascites formation

Liver disease
Peritoneal carcinomatosis
Heart disease
Pancreatitis
Infectious disease (e.g., tuberculosis, chlamydia infection)
Miscellaneous (e.g., post-surgery, autoimmune disease, renal disease)

Ascites is not limited to liver disease, so that other causes of ascites formation should be considered in every patient with ascites. Ascites biomarkers discussed in this chapter refer to ascites caused by liver disease.

Table 2 Inflammatory ascites biomarkers

Adenosine deaminase
Alkaline phosphatase
Ascites pH
Bacterial DNA
Calprotectin
Carcinoembryogenic antigen (CEA)
Complement factors
Glucose
Interferon gamma-induced protein 10 kDa
Interleukin-1 beta
Interleukin-8
Interleukin-6
Interleukin-10
Lactate dehydrogenase (LDH)
Lactoferrin
Leukocyte esterase activity (measured by urinary reagent strips)
Macrophage inhibitory protein-1 beta
Neutrophil gelatinase-associated lipocalin (NGAL)
Opsonic activity
Polymorphonuclear cell count
Procalcitonin
Total protein
Tumor necrosis factor-alpha (TNF-alpha) and its receptors

This table provides an overview about the ascites biomarkers which are discussed in this chapter.

Methods

The molecular diversity of biomarkers that have been studied in the ascites is considerable, including cell counts, proteins, nucleic acid, and enzyme activity. However, analysis of biomarkers in the ascites relies in most cases on tests developed for other biological materials, mostly blood. Therefore, the methods used are in

Table 3 Factors potentially influencing ascites biomarkers

Etiology of liver disease
Severity of liver disease
Complications of liver cirrhosis: spontaneous bacterial peritonitis, hepatorenal syndrome, portal vein thrombosis
Medication, e.g., antibiotics including rifaximin, diuretics
Concomitant conditions: secondary peritonitis, peritoneal carcinomatosis

These factors should be taken into account when assessing novel ascites biomarkers, because they are known to influence the levels of ascites biomarkers.
References are given in the main text

Table 4 Established biomarkers in ascites fluid of patients with chronic liver disease

Biomarker	Indication	Significance	Limitations
PMN cell count	Routine	>250 cells/mm^3: indicates SBP	Bloody ascites, secondary peritonitis, malignant ascites
Total protein	Routine	Below 10 (15) g/L: indicates high risk for SBP	Accuracy may be limited, e.g., in patients receiving diuretics
Lactate dehydrogenase (LDH), glucose	Suspicion of secondary peritonitis	Elevated LDH and glucose <50 mg/dL: may indicate secondary peritonitis in conjunction with polymicrobial growth in ascites cultures and total protein >10 g/L	Inferior to abdominal CT scan
Adenosine deaminase	Suspicion of tuberculosis	Elevated in tuberculosis	Limited sensitivity in concomitant liver disease

This table shows ascites biomarkers that are used in clinical practice.
References are given in the main text.
PMN polymorphonuclear, *SBP* spontaneous bacterial peritonitis

general not particular to ascites. One exception of a test form developed especially for cirrhotic ascites is a reagent strip (Mendler et al. 2010). This strip, however, is derived from reagent strips applied to urine. Heterogeneity of methods is particularly broad for analyzing bacterial DNA in the ascites. The amount of ascites that is centrifuged to gain the cell pellet from which the DNA is extracted, the method of extraction, and the primers vary considerably. Since bacterial DNA is not determined in clinical practice, standardization of methods has not become necessary.

Established Biomarkers in the Ascites Fluid

Determination of ascites biomarkers is common in cirrhotic patients (Table 4). Biomarkers that are used in clinical practice will be discussed in detail in this section.

PMN Count

The diagnostic marker used most commonly in clinical practice is the ascites PMN count. SBP is diagnosed per definition if the PMN count exceeds 250 cells/mm^3 based on current international guidelines (European Association for the Study of the Liver 2010; Runyon and AASLD 2013). The cutoff has been chosen due to its high sensitivity for culture-positive peritonitis but has not been validated for culture-negative SBP (European Association for the Study of the Liver 2010; Wiest et al. 2012). Since SBP, in contrast to other forms of peritonitis, is often asymptomatic, it is difficult to establish a diagnostic threshold for SBP based on clinical grounds. Therefore, although the cutoff of 250 PMN/mm^3 is widely accepted, it must be borne in mind that the number of false-positive or false-negative results is not known (Wiest et al. 2012). A decrease of the PMN count by 25% after 48 h of treatment is often used as indicator for treatment response (European Association for the Study of the Liver 2010; Wiest et al. 2012; Runyon and AASLD 2013).

Opsonic Activity and Total Protein Content

Once SBP develops, prognosis of patients with liver cirrhosis and ascites is poor. Therefore, attempts have been made to identify patients at high risk for SBP. Low ascites total protein has been proposed as risk factor for SBP so that current guidelines recommend to measure total protein in ascites. Patients with a total ascites protein content below 15 g/L may benefit from primary antibiotic prophylaxis to prevent SBP (European Association for the Study of the Liver 2010; Runyon and AASLD 2013).

Ascites protein content correlates with the quantity of ascites complement factors (Runyon et al. 1985), the concentration of immunoglobulin G and A in the ascites (García-Díaz et al. 1995), and opsonic activity of ascites (Runyon et al. 1985). In cirrhotic ascites, opsonic and bactericidal activity is markedly reduced compared to serum from cirrhotic patients or peritoneal fluid obtained during surgery from non-cirrhotic patients, which has been attributed to the low complement levels found in ascites (Simberkoff et al. 1978). Multivariate analysis showed in different studies that low ascites complement levels or low opsonic activity, but not total protein content, were independently associated with occurrence of SBP (Mal et al. 1991; Andreu et al. 1993). Still, measurement of total protein is established in clinical routine, so that ascites total protein has been tested as biomarker for the risk to acquire SBP in several studies dating from 1986 to 1993. A first study reported a significantly increased risk for SBP if ascites total protein was below 10 g/L among 82 patients with cirrhotic ascites, of whom 17 developed SBP (Runyon 1986). Another study assessed the risk for recurrence of SBP in 75 cirrhotic patients. Again, total ascites protein below 10 g/L was found to be a predictive factor for SBP (Titó et al. 1988). Similar results were obtained in two studies analyzing risk

factors for a first episode of SBP in 127 and 110 patients with liver cirrhosis and ascites, respectively (Llach et al. 1992; Andreu et al. 1993).

Since these studies indicated that patients at high risk for SBP might be identified by low ascites protein content, several studies analyzed if antibiotic prophylaxis might prevent SBP in such patients. However, a threshold of 15 g/L ascites total protein was chosen to separate patients into a low and high ascites protein group. In 63 patients with low ascites protein randomized to receive norfloxacin or not, SBP occurred in 23% of patients without prophylaxis but in no patient taking norfloxacin ($p < 0.05$) (Soriano et al. 1991). In another trial assessing prophylaxis with norfloxacin for 6 months in patients with low ascites protein and without prior SBP, incidence of SBP was reduced by norfloxacin, but the reduction failed to reach statistical significance (Grangé et al. 1998). Another study evaluated primary antibiotic prophylaxis in 68 patients with low ascites protein plus either signs of terminal liver cirrhosis or impaired kidney function (Fernández et al. 2007). In this group of patients, antibiotic prophylaxis significantly reduced the incidence of SBP. Finally, from a study comprising 100 patients with low ascites protein randomized to primary prophylaxis with ciprofloxacin or no prophylaxis, a nonsignificant reduction of SBP (Terg et al. 2008) was reported. In summary, primary antibiotic prophylaxis seems to be effective in preventing SBP at least in patients with terminal liver cirrhosis and low ascites protein. However, study results are divergent regarding the effect size of antibiotic prophylaxis (European Association for the Study of the Liver 2010; Runyon and AASLD 2013), which may at least in part be attributed to a low power of the available studies to detect a statistical significant difference.

In spite of this promising application of a simple biomarker to clinical intervention, some more recent studies, which aimed to detect other risk factors for SBP, failed to replicate an association between low ascites protein content and SBP (Schwabl et al. 2015; Terg et al. 2015). Therefore, a post hoc analysis of two German cohorts comprising 347 and 336 patients with liver cirrhosis and ascites was performed. No association between levels of ascites protein and occurrence of SBP was found (Bruns et al. 2015). These results put into question if ascites total protein can be considered a reliable prognostic marker for SBP in every clinical setting.

The reason for the diverging results of studies dating some years back and more recent analyses concerning the prognostic significance of low ascites protein is not clear. Of note, the amount of total ascites protein is not altered by the presence of SBP (Runyon and Hoefs 1985). However, little is known if ascites total protein is changed by factors such as increasing severity of liver disease over time and acute or chronic liver failure, respectively. Several studies indicate that ascites protein as surrogate marker might not correlate closely enough to the direct biomarker, opsonic activity (Mal et al. 1991; Andreu et al. 1993). In line with this hypothesis, diuretic treatment increases opsonic activity in the ascites to a greater extent than total protein levels (Runyon and Van Epps 1986). Opsonic activity and complement factors in the ascites were enhanced by diuretic treatment but not by paracentesis in two randomized trials (Runyon et al. 1989; Ljubicić et al. 1994). Diuretic treatment increased

local antimicrobial activity of the ascites also in the subgroup of patients who had survived an episode of SBP (Runyon et al. 1992). A randomized controlled trial comprising 80 patients addressed the issue if ascites treatment with diuretics compared to paracentesis decreases the risk for SBP. Rates of SBP were comparable in both treatment arms (Solà et al. 1995). However, patients in the paracentesis arm were additionally treated with diuretics, so that the effect of diuretics could not be properly assessed by this study. In conclusion, experimental data suggest that diuretic treatment may decrease the risk for SBP by enhancing antibacterial activity, which may be most relevant for patients with low ascites protein. However, clinical data are not sufficient to prove this hypothesis, since diuretic treatment has rarely been assessed separately as a risk factor for SBP.

In summary, total protein in ascites is a surrogate marker for bactericidal activity of the ascites. Low ascites protein has been proposed as indicator for an increased SBP risk. However, changes in treatment or epidemiology seem to have uncoupled this surrogate marker from the underlying risk factor. This underlines the need to detect new biomarkers in the ascites.

Markers to Distinguish Secondary Peritonitis from SBP

SBP is a form of primary peritonitis. Primary peritonitis is very rare in patients without a predisposing condition such as liver cirrhosis with ascites. In the absence of such predisposing factors, peritonitis is usually secondary and due to an intra-abdominal source of infection, e.g., bowel perforation or abscess formation. Treatment is fundamentally different: while SBP is treated with antibiotics and intravenous substitution of albumin (European Association for the Study of the Liver 2010; Runyon and AASLD 2013), surgery is necessary in the case of secondary peritonitis. Secondary peritonitis may mimic SBP, so that a secondary form of peritonitis has to be ruled out in any patient with suspected SBP. Several ascites biomarkers have been proposed to facilitate the distinction between SBP and secondary peritonitis. Ascites total protein above 10 g/L, ascites lactate dehydrogenase above the norm for serum, ascites glucose below 50 mg/dL, and simultaneous detection of several different microorganisms in ascites cultures provide hints toward a diagnosis of secondary peritonitis with high sensitivity (Akriviadis and Runyon 1990; Soriano et al. 2010). In addition, ascites alkaline phosphatase levels greater than 240 units/L or levels of carcinoembryogenic antigen (CEA) above 5 ng/mL have been proposed to accurately indicate secondary peritonitis with greater specificity than the aforementioned biomarkers (Wu et al. 2001). In contrast to European guidelines, American guidelines recommend testing for total protein, glucose, and lactate dehydrogenase if secondary peritonitis is suspected (Runyon and AASLD 2013). However, since computed tomography (CT) scanners have become widely available, diagnosis of secondary peritonitis should be based on imaging techniques, as recommended by European guidelines (European Association for the Study of the Liver 2010).

Table 5 Ascites biomarkers proposed as diagnostic makers for spontaneous bacterial peritonitis

Biomarker	Sensitivity	Specificity	Limitation
Polymorphonuclear cell count	Gold standard		
Interferon gamma-induced protein 10 kDa	93%	87%	More studies needed
Interleukin-6	98%	78%	More studies needed
Lactoferrin	96%	97%	More studies needed
Leukocyte esterase activity (measured by special urine test strips)	100%	58%	More studies needed; chylous/bloody ascites
Ratio calprotectin/total protein	93%	79%	More studies needed
Tumor necrosis factor-alpha	91%	83%	More studies needed

This table shows ascites biomarkers that may be alternative diagnostic tools for spontaneous bacterial peritonitis but have not become part of clinical routine.
References are given in the main text

Adenosine Deaminase

Although nowadays rare in Western countries, tuberculosis has remained an important cause of peritonitis in certain areas of the world (Sanai and Bzeizi 2005). Adenosine deaminase activity, which is increased in T lymphocytes upon stimulation, has been proposed as a biomarker in ascites to detect tuberculous peritonitis (Tao et al. 2014). However, analysis of adenosine deaminase activity in 368 ascites specimens from Western patients indicated that the sensitivity of the test was limited, which was attributed to a high proportion of patients with concomitant liver cirrhosis (Hillebrand et al. 1996).

Ascites Biomarkers That Have Not Yet Been Introduced into Clinical Practice

A major focus of research has been to increase the detection rate of microorganisms in infected ascites by molecular techniques. Results obtained by analysis of ascites bacterial DNA content will be discussed first. Then, several inflammatory biomarkers that have been studied experimentally in the ascites will briefly be reported. A major interest has been to find new diagnostic markers that can be analyzed rapidly also in clinical settings where a PMN cell count of the ascites is not readily available. Not surprisingly, most of these studies have used proteins secreted by neutrophils. These potential new diagnostic biomarkers will be discussed in more detail. Markers that have been proposed as diagnostic test for SBP in large cohorts of patients with liver cirrhosis are summarized in Table 5.

Bacterial DNA

Blood-culture bottles immediately filled with ascites at the patient's bedside are the standard tool to detect causative microorganisms in SBP. However, positive culture results are only achieved in about 50% of cases (European Association for the Study of the Liver 2010; Runyon and AASLD 2013). By contrast, only 40% of patients with bacterial growth in ascites but without elevated ascites PMN counts, the so-called bacterascites, seem to need antibiotic therapy, because the majority of patients may eliminate the microorganism spontaneously (Runyon 1990). Given this limited accuracy of conventional ascites cultures for the diagnosis of SBP, detection of bacterial DNA by molecular amplification techniques has been investigated extensively as alternative means to identify the microorganism causing SBP. The feasibility of such an approach was first suggested by Such and colleagues, who detected the same bacterial DNA, mostly from *Escherichia coli*, simultaneously in blood and ascites in 9 out of 28 patients with liver cirrhosis and ascites (Such et al. 2002). Later, Frances and colleagues found bacterial DNA in all 13 patients with culture-negative SBP that were included in their analysis. Sequencing the bacterial DNA, they found a similar spectrum of bacteria as detected by conventional cultures. In nine patients with culture-positive SBP, only a single identified microorganism was discordant between conventional and PCR-based analysis (Francés et al. 2008). However, bacterial DNA was detected in both studies in about one third of patients without infection, indicating low specificity of this method. Subsequent studies using different technologies failed to reliably identify the causative microorganism in culture-negative and culture-positive SBP, thus confirming the low specificity of molecular amplification methods (Vieira et al. 2007; Sugihara et al. 2009; Appenrodt et al. 2010; Soriano et al. 2011; Krohn et al. 2014; Mortensen et al. 2014). These results indicate that analysis of bacterial DNA in the ascites is complex. The presence of bacterial DNA is frequent but does not correlate closely with concomitant infection by viable and virulent bacteria. In support of this hypothesis, when ascites of 25 patients was subjected to 16S rRNA pyrosequencing of viable bacteria, most ascites samples were found to contain DNA of a vast range of bacteria. In addition, severity of liver disease had a major impact on microbial ascites composition (Rogers et al. 2013). In order to increase sensitivity and specificity, a pilot study used in situ hybridization of bacterial DNA phagocytized by ascites leukocytes (Enomoto et al. 2012). Bacterial DNA was found in 10/11 cases with SBP, including eight culture-negative SBP cases but in none of the 40 samples without SBP. However, identification of the bacterial species was limited, and the results have to be confirmed in a larger trial.

Although analysis of bacterial DNA in ascites has important limitations for the diagnosis of SBP, it may have prognostic impact. In a study involving 156 patients, increased 1-year mortality in patients with noninfected ascites was found if bacterial DNA was detected (Zapater et al. 2008). However, the presence of bacterial DNA did not predict development of SBP. In another study using quantitative PCR in 25 patients with liver cirrhosis, patients who died or developed SBP within 6 months had significantly higher bacterial DNA burden in their ascites at index paracentesis

(Fagan et al. 2015). Due to its limitations, bacterial DNA has not yet been introduced as a routine test for patients with liver cirrhosis and ascites.

Inflammatory Cytokines

Several cytokines and chemokines are increased in ascites of patients with SBP compared to patients without SBP. These include macrophage inhibitory protein-1 beta (Lesińska et al. 2014), interleukin-1 beta (Rodríguez-Ramos et al. 2001), interleukin-8 (Martínez-Brú et al. 1999), interleukin-10 (Kim et al. 2007), tumor necrosis factor-alpha (TNF-alpha) (Rodríguez-Ramos et al. 2001; Kiyici et al. 2006; Abdel-Razik et al. 2015), and the soluble receptors of TNF-alpha (Andus et al. 1992).

Concerning interleukin-6, one study in 20 patients, which found no difference in the levels of interleukin-6 (Spahr et al. 2001), is in contrast to four studies involving 63 patients (Pruimboom et al. 1995), 37 patients (Propst et al. 1993), 47 patients (Rodríguez-Ramos et al. 2001), and 425 patients (Abdel-Razik et al. 2015), respectively, which found significantly increased interleukin-6 levels during SBP. A small study comprising six patients with and six patients without previous episodes of SBP suggested that patients with previous SBP had lower interleukin-6 levels in the ascites during the absence of infection (Souza et al. 2003).

Recently, interferon gamma-induced protein 10 kDa, interleukin-6, and TNF-alpha in ascites have been reported as potential diagnostic parameters for SBP in a large study from Egypt on 425 patients with cirrhosis, 61 of whom had SBP (Abdel-Razik et al. 2015). However, cirrhosis was almost exclusively due to viral hepatitis in this study, and important subgroups were excluded, for example, patients with sepsis or hyperlipidemia. Therefore, the results can only be transferred with caution to real-life cohorts in Western countries.

Interestingly, a randomized study comparing 15 patients with SBP who received antibiotics and albumin to 15 patients with SBP who received only antibiotics suggested that intravenous treatment with albumin results in decreased ascites levels of TNF-alpha and interleukin-6 (Chen et al. 2009).

Procalcitonin

Serum procalcitonin is a biomarker which is established in clinical routine and which is supposed to be specific for bacterial infections (Schuetz et al. 2011). Ascites procalcitonin has been evaluated as indicator of SBP in patients with liver cirrhosis. However, in an analysis of ten patients with and ten patients without SBP, procalcitonin was only increased in the serum of patients with SBP but not in ascites (Spahr et al. 2001). Another study including 32 patients with decompensated liver cirrhosis, of whom 10 had SBP, confirmed that levels of ascites procalcitonin do not distinguish between patients with and without SBP (Lesińska et al. 2014).

pH

Ascites pH has been proposed as diagnostic marker for spontaneous bacterial peritonitis (Gitlin et al. 1982). However, current guidelines do not recommend testing ascites pH (Runyon and AASLD 2013), because ascites PMN count is a better marker for SBP (Scemama-Clergue et al. 1985; Pinzello et al. 1986).

Urinary Reagent Strips

Since a PMN cell count cannot be rapidly obtained in every clinical setting in which diagnostic paracentesis is performed due to suspicion of SBP, alternative tests that can be carried out at the bedside have been investigated. A first approach used colorimetric reagent strips which are normally applied to urine for diagnosis of urinary tract infections and which measure leukocyte esterase activity semiquantitatively. However, a large review including 19 studies concluded that this bedside test showed low sensitivity and yielded a high proportion of false-negative results (Nguyen-Khac et al. 2008). Therefore, a new reagent strip calibrated to ascites was developed and evaluated in a study which involved ascites samples from 32 patients without and from 26 patients with SBP as well as more than 1,000 experiments with diluted and spiked ascites (Mendler et al. 2010). The area under the receiver operator curve (AUROC) was 0.92, sensitivity 100%, and specificity 58%. However, due to its colorimetric scale, the test cannot be applied to opaque ascites, such as bloody or chylous ascites. In addition, test performance has not been confirmed in a large patient cohort.

Neutrophil Gelatinase-Associated Lipocalin (NGAL)

NGAL is a 24kDA antimicrobial protein expressed in neutrophils, renal tubular cells, and hepatocytes, among others. NGAL has been mainly studied as biomarker for acute kidney injury, since its production is upregulated in the nephron upon damage and secreted into the urine (Singer et al. 2013).

NGAL was analyzed as biomarker for bacterial peritonitis in one study comprising 111 ascites samples from patients with and without liver disease (Lippi et al. 2013). Twenty-six samples were classified as bacterial peritonitis based on a PMN cell count above 250 cells/mm^3. NGAL levels correlated with ascites PMN count ($r = 0.77$; $p < 0.001$). AUROC was 0.89 for diagnosis of SBP. A cutoff of 120 ng/mL resulted in a sensitivity of 96% and a specificity of 75%. Test performance was further improved when lactate dehydrogenase (LDH) was used as additional marker.

A shortcoming of this study is that the patients whose ascites specimens were analyzed had poorly been characterized. Samples were included if they represented new onset ascites from a hepatology and infectious disease unit and if they were not related to malignancy or peritoneal dialysis. Thus, it is likely that most, but

presumably not all patients suffered from liver cirrhosis. In addition, association between ascites NGAL levels and the clinical course of SBP or the incidence of hepatorenal syndrome was not assessed. Taken together, NGAL in ascites is a promising biomarker for SBP but has to be evaluated further till it can be used in clinical practice.

Lactoferrin

Lactoferrin is another antimicrobial protein of about 80 kDa molecular weight which is stored in neutrophils. In feces, lactoferrin is a biomarker for diagnosis and monitoring disease activity of inflammatory bowel disease (Wright et al. 2014). The use of ascites lactoferrin for diagnosis of SBP was assessed in 218 ascites specimens from 148 patients with liver cirrhosis (Parsi et al. 2008). SBP was diagnosed in 22 samples by a PMN cell count above 250 cells/mm^3. When a diagnostic threshold of 242 ng/mL lactoferrin was used, AUROC was 0.98, sensitivity 96%, and specificity 97%. In addition, lactoferrin levels decreased in all patients who responded to antibiotic treatment. The results of this study indicate that ascites lactoferrin might be a useful biomarker to detect SBP and to assess treatment response; however, these results as well as the cutoff of 242 ng/mL have to be validated.

Calprotectin

Calprotectin is an acute phase inflammatory protein of 36 kDa. It is secreted mainly by PMN and has antimicrobial, antiproliferative, and regulatory functions. It is very stable in clinical specimens, favoring its applicability as biomarker (Johne et al. 1997). Like lactoferrin, fecal calprotectin levels are determined to diagnose and monitor inflammatory bowel disease (Wright et al. 2014). Three studies evaluated ascites calprotectin as biomarker in liver cirrhosis.

In a first study comprising ascites samples from 10 patients with malignant ascites and 65 patients with ascites due to alcoholic liver cirrhosis (Homann et al. 2003), calprotectin levels in plasma and ascites correlated significantly ($r = 0.60$; $p < 0.0001$). Ascites calprotectin levels were found to be markedly increased in patients with malignant ascites compared to patients with ascites due to liver cirrhosis. Interestingly, ascites calprotectin levels above the upper quartile (>112 ng/mL) predicted poor survival in patients with liver cirrhosis even if 15 patients with bacterial infections were excluded from the analysis. Samples with SBP were not assessed separately.

A second study investigated in 130 ascites specimens from 71 patients if ascites calprotectin could substitute for a PMN cell count above 250 cells/mm^3 (Burri et al. 2013). However, the diseases causing ascites were heterogeneous, so that only 4 of the 19 samples with elevated PMN cell counts were classified as SBP. The strength of this study is that calprotectin levels were not only measured by

conventional enzyme-linked immunosorbent assay (ELISA) but also by a point-of-care test. Correlation between calprotectin levels and PMN cell counts in ascites was good ($r = 0.48$; $p < 0.001$).

These findings triggered a third study (Lutz et al. 2015b) which evaluated PMN counts and calprotectin levels in 120 ascites samples from 100 patients with liver cirrhosis and ascites, including 27 samples with SBP. As expected, ascites calprotectin correlated closely with ascites PMN cell counts ($r = 0.59$; $p < 0.001$). Of note, increased calprotectin levels were measured in the samples with SBP but not in those from patients with bacterascites. In addition, calprotectin levels in noninfected ascites were higher in patients with alcoholic compared to viral liver disease, in patients with Child-Pugh stage B versus C cirrhosis and in patients with portal vein thrombosis. However, differences between the subgroups did not reach the levels associated with SBP. If a diagnostic threshold of 36.1 ng/mL was chosen to ensure a sensitivity of 90%, specificity was only 51%. However, test performance could be improved by calculating the ratio of ascites calprotectin to ascites total protein, which probably reflects intraperitoneal calprotectin production. The calprotectin to total protein ratio resulted in an AUROC of 0.93, a sensitivity of 93%, and a specificity of 79% for a cutoff of 5.24 (applying a correction factor of 10^{-6}). In addition, a high calprotectin to total protein ratio was associated with poor prognosis in patients with SBP.

Thus, ascites calprotectin may be an interesting diagnostic and prognostic biomarker when confirmed by further evaluation.

Potential Applications of Inflammatory Ascites Biomarkers to Assess Prognosis, Other Diseases, or Conditions

The potential of ascites biomarkers to predict prognosis has been discussed in detail in the subsections regarding the respective biomarkers. Ascites biomarkers that may predict the risk for SBP are listed in Table 6. Table 7 shows ascites biomarkers that have been associated with mortality. In short summary of the preceding subsections of this chapter, low ascites total protein (defined as either below 15 g/L or below 10 g/L) has been associated with a high risk for acquiring SBP (Runyon 1986; European Association for the Study of the Liver 2010). However, the replicability of low ascites protein as risk factor for SBP in more recent studies has been limited. Complement levels and opsonic activity in the ascites may be more important prognostic factors but have not been validated in clinical practice. Several studies have been carried out to assess if primary antibiotic prophylaxis can prevent SBP in patients with low ascites protein. These studies failed to show a uniform benefit (European Association for the Study of the Liver 2010). Therefore, international guidelines recommend primary prophylaxis with norfloxacin only in patients with low ascites protein if they present with signs of terminal liver disease (European Association for the Study of the Liver 2010).

Ascites calprotectin levels have been associated with mortality of patients with and without SBP (Homann et al. 2003; Lutz et al. 2015b). However, these findings

Table 6 Ascites biomarkers potentially indicating a high risk for spontaneous bacterial peritonitis

Biomarker	Prognostic for SBP when	Limitations
Opsonic activity	Low	Not established in clinical practice
Complement factors	Low	Not established in clinical practice
Total protein	Low (<10 g/L)	Established in clinical practice but surrogate marker with potentially limited accuracy

This table shows ascites biomarkers that have been proposed to indicate an increased risk to acquire spontaneous bacterial peritonitis.
References are given in the main text

Table 7 Ascites biomarkers associated with mortality

Biomarker	Context
Bacterial DNA	High mortality if (high levels of) bacterial DNA are detected
Calprotectin	Poor prognosis in patients with alcoholic liver disease without bacterial infections
Ratio of calprotectin/total protein	Poor short-term prognosis in patients with spontaneous bacterial peritonitis
Tumor necrosis factor-alpha, interleukin-6, neopterin	High mortality in spontaneous bacterial peritonitis

This table shows ascites biomarkers that have been proposed to indicate a high mortality.
References are given in the main text

have to be validated in larger cohorts and are not yet part of clinical practice. Furthermore, levels of interleukin-6, neopterin, and tumor necrosis factor-alpha have been suggested to predict the prognosis of patients with SBP (Propst et al. 1993). In addition, the presence of bacterial DNA and the amount of bacterial DNA in the ascites have been associated with mortality (Zapater et al. 2008; Fagan et al. 2015).

An important complication in patients with SBP associated with high mortality is acute kidney failure (Tandon and Garcia-Tsao 2011). In a study comprising 52 patients with SBP, ascites levels of TNF-alpha and interleukin-6 were associated with development of acute kidney failure (Navasa et al. 1998). Another study reported levels of ascites nitric oxide metabolites as predictors of renal failure in patients with SBP (Such et al. 2004). None of these markers has been introduced into clinical practice.

Ascites biomarkers may be important in patients without liver disease, for example in patients on peritoneal dialysis (Krediet et al. 2009) or with peritoneal carcinomatosis (Gulyás et al. 2001), but this is out of the scope of this chapter. Most biomarkers referred to in this chapter are used as diagnostic or prognostic parameters for other diseases in other biological materials, e.g., fecal calprotectin or fecal lactoferrin in inflammatory bowel disease (Wright et al. 2014).

Summary Points

- Ascites offers a unique opportunity to easily access body fluid apart from blood. Future research will show if ascites biomarkers may be superior to blood biomarkers in liver disease in other conditions than spontaneous bacterial peritonitis.
- As recommended by international guidelines, an ascites polymorphonuclear cell count above 250 cells/mm^3 establishes the diagnosis of spontaneous bacterial peritonitis in patients with liver cirrhosis if secondary peritonitis is excluded.
- Alternative markers for diagnosis of spontaneous bacterial peritonitis such as calprotectin, lactoferrin, or urinary test sticks rely on polymorphonuclear cell activity but are not used outside of clinical studies.
- In addition, ascites calprotectin may confer prognostic information in patients with and without spontaneous bacterial peritonitis.
- Total protein in the ascites is a surrogate marker for opsonic factors. Low levels may reflect an increased risk for spontaneous bacterial peritonitis in a subgroup of patients that has to be defined more closely.
- The presence of bacterial DNA in the ascites is evidence for bacterial translocation. However, determination of bacterial DNA in ascites does not give additional clinical information beyond polymorphonuclear cell count and conventional microbiological culture for diagnosis of spontaneous bacterial peritonitis.

References

Abdel-Razik A, Mousa N, Elbaz S, Eissa M, Elhelaly R, Eldars W. Diagnostic utility of interferon gamma-induced protein 10 kDa in spontaneous bacterial peritonitis: single-center study. Eur J Gastroenterol Hepatol. 2015;27:1087–93.

Akriviadis EA, Runyon BA. Utility of an algorithm in differentiating spontaneous from secondary bacterial peritonitis. Gastroenterology. 1990;98:127–33.

Andreu M, Sola R, Sitges-Serra A, Alia C, Gallen M, Vila MC, et al. Risk factors for spontaneous bacterial peritonitis in cirrhotic patients with ascites. Gastroenterology. 1993;104:1133–8.

Andus T, Gross V, Holstege A, Ott M, Weber M, David M, et al. High concentrations of soluble tumor necrosis factor receptors in ascites. Hepatol Baltim MD. 1992;16:749–55.

Appenrodt B, Lehmann LE, Thyssen L, Gentemann M, Rabe C, Molitor E, et al. Is detection of bacterial DNA in ascitic fluid of clinical relevance? Eur J Gastroenterol Hepatol. 2010;22:1487–94.

Bruns T, Lutz P, Stallmach A, Nischalke HD. Low ascitic fluid protein does not indicate an increased risk for spontaneous bacterial peritonitis in current cohorts. J Hepatol. 2015;63:527–8.

Burri E, Schulte F, Muser J, Meier R, Beglinger C. Measurement of calprotectin in ascitic fluid to identify elevated polymorphonuclear cell count. World J Gastroenterol WJG. 2013;19:2028–36.

Chen T-A, Tsao Y-C, Chen A, Lo G-H, Lin C-K, Yu H-C, et al. Effect of intravenous albumin on endotoxin removal, cytokines, and nitric oxide production in patients with cirrhosis and spontaneous bacterial peritonitis. Scand J Gastroenterol. 2009;44:619–25.

Enomoto H, Inoue S, Matsuhisa A, Aizawa N, Imanishi H, Saito M, et al. Development of a new in situ hybridization method for the detection of global bacterial DNA to provide early evidence of a bacterial infection in spontaneous bacterial peritonitis. J Hepatol. 2012;56:85–94.

European Association for the Study of the Liver. EASL clinical practice guidelines on the management of ascites, spontaneous bacterial peritonitis, and hepatorenal syndrome in cirrhosis. J Hepatol. 2010;53:397–417.

Fagan KJ, Rogers GB, Melino M, Arthur DM, Costello M-E, Morrison M, et al. Ascites bacterial burden and immune cell profile are associated with poor clinical outcomes in the absence of overt infection. PLoS ONE. 2015;10, e0120642.

Fernández J, Navasa M, Planas R, Montoliu S, Monfort D, Soriano G, et al. Primary prophylaxis of spontaneous bacterial peritonitis delays hepatorenal syndrome and improves survival in cirrhosis. Gastroenterology. 2007;133:818–24.

Francés R, Zapater P, González-Navajas JM, Muñoz C, Caño R, Moreu R, et al. Bacterial DNA in patients with cirrhosis and noninfected ascites mimics the soluble immune response established in patients with spontaneous bacterial peritonitis. Hepatol Baltim MD. 2008;47:978–85.

García-Díaz M, Alcalde M, de Sande F, Romero J, Sánchez-Risco P, Pijierro A, et al. Low protein concentration in cirrhotic ascites is related to low ascitic concentrations of immunoglobulins G and A. Eur J Gastroenterol Hepatol. 1995;7:963–9.

Gitlin N, Stauffer JL, Silvestri RC. The pH of ascitic fluid in the diagnosis of spontaneous bacterial peritonitis in alcoholic cirrhosis. Hepatol Baltim MD. 1982;2:408–11.

Grangé JD, Roulot D, Pelletier G, Pariente EA, Denis J, Ink O, et al. Norfloxacin primary prophylaxis of bacterial infections in cirrhotic patients with ascites: a double-blind randomized trial. J Hepatol. 1998;29:430–6.

Gulyás M, Kaposi AD, Elek G, Szollár LG, Hjerpe A. Value of carcinoembryonic antigen (CEA) and cholesterol assays of ascitic fluid in cases of inconclusive cytology. J Clin Pathol. 2001;54:831–5.

Hillebrand DJ, Runyon BA, Yasmineh WG, Rynders GP. Ascitic fluid adenosine deaminase insensitivity in detecting tuberculous peritonitis in the United States. Hepatol Baltim MD. 1996;24:1408–12.

Homann C, Christensen E, Schlichting P, Philipsen EK, Graudal NA, Garred P. Ascites fluid and plasma calprotectin concentrations in liver disease. Scand J Gastroenterol. 2003;38:415–20.

Johne B, Fagerhol MK, Lyberg T, Prydz H, Brandtzaeg P, Naess-Andresen CF, et al. Functional and clinical aspects of the myelomonocyte protein calprotectin. Mol Pathol MP. 1997; 50:113–23.

Kim JK, Chon CY, Kim JH, Kim YJ, Cho JH, Bang SM, et al. Changes in serum and ascitic monocyte chemotactic protein-1 (MCP-1) and IL-10 levels in cirrhotic patients with spontaneous bacterial peritonitis. J Interferon Cytokine Res Off J Int Soc Interferon Cytokine Res. 2007;27:227–30.

Kiyici M, Nak SG, Budak F, Gurel S, Oral B, Dolar E, et al. Lymphocyte subsets and cytokines in ascitic fluid of decompensated cirrhotic patients with and without spontaneous ascites infection. J Gastroenterol Hepatol. 2006;21:963–9.

Krediet RT, Sampimon DE, Vlijm A, Coester AM, Struijk DG, Smit W. Biological markers in the peritoneal dialysate effluent: are they useful. Contrib Nephrol. 2009;163:54–9.

Krohn S, Böhm S, Engelmann C, Hartmann J, Brodzinski A, Chatzinotas A, et al. Application of qualitative and quantitative real-time PCR, direct sequencing, and terminal restriction fragment length polymorphism analysis for detection and identification of polymicrobial 16S rRNA genes in ascites. J Clin Microbiol. 2014;52:1754–7.

Lesińska M, Hartleb M, Gutkowski K, Nowakowska-Duława E. Procalcitonin and macrophage inflammatory protein-1 beta (MIP-1β) in serum and peritoneal fluid of patients with decompensated cirrhosis and spontaneous bacterial peritonitis. Adv Med Sci. 2014;59:52–6.

Lippi G, Caleffi A, Pipitone S, Elia G, Ngah A, Aloe R, et al. Assessment of neutrophil gelatinase-associated lipocalin and lactate dehydrogenase in peritoneal fluids for the screening of bacterial peritonitis. Clin Chim Acta Int J Clin Chem. 2013;418:59–62.

Ljubicić N, Bilić A, Kopjar B. Diuretics vs. paracentesis followed by diuretics in cirrhosis: effect on ascites opsonic activity and immunoglobulin and complement concentrations. Hepatol Baltim MD. 1994;19:346–53.

Llach J, Rimola A, Navasa M, Ginès P, Salmerón JM, Ginès A, et al. Incidence and predictive factors of first episode of spontaneous bacterial peritonitis in cirrhosis with ascites: relevance of ascitic fluid protein concentration. Hepatol Baltim MD. 1992;16:724–7.

Lutz P, Nischalke HD, Strassburg CP, Spengler U. Spontaneous bacterial peritonitis: the clinical challenge of a leaky gut and a cirrhotic liver. World J Hepatol. 2015a;7:304.

Lutz P, Pfarr K, Nischalke HD, Krämer B, Goeser F, Glässner A, et al. The ratio of calprotectin to total protein as a diagnostic and prognostic marker for spontaneous bacterial peritonitis in patients with liver cirrhosis and ascites. Clin Chem Lab Med CCLM FESCC. 2015b;53:2031–9.

Mal F, Huu TP, Bendahou M, Trinchet JC, Garnier M, Hakim J, et al. Chemoattractant and opsonic activity in ascitic fluid. A study in 47 patients with cirrhosis or malignant peritonitis. J Hepatol. 1991;12:45–9.

Martínez-Brú C, Gómez C, Cortés M, Soriano G, Guarner C, Planella T, et al. Ascitic fluid interleukin-8 to distinguish spontaneous bacterial peritonitis and sterile ascites in cirrhotic patients. Clin Chem. 1999;45:2027–8.

Mendler MH, Agarwal A, Trimzi M, Madrigal E, Tsushima M, Joo E, et al. A new highly sensitive point of care screen for spontaneous bacterial peritonitis using the leukocyte esterase method. J Hepatol. 2010;53:477–83.

Mortensen C, Jensen JS, Hobolth L, Dam-Larsen S, Madsen BS, Andersen O, et al. Association of markers of bacterial translocation with immune activation in decompensated cirrhosis. Eur J Gastroenterol Hepatol. 2014;26:1360–6.

Navasa M, Follo A, Filella X, Jiménez W, Francitorra A, Planas R, et al. Tumor necrosis factor and interleukin-6 in spontaneous bacterial peritonitis in cirrhosis: relationship with the development of renal impairment and mortality. Hepatol Baltim MD. 1998;27:1227–32.

Nguyen-Khac E, Cadranel JF, Thevenot T, Nousbaum JB. Review article: the utility of reagent strips in the diagnosis of infected ascites in cirrhotic patients. Aliment Pharmacol Ther. 2008;28:282–8.

Parsi MA, Saadeh SN, Zein NN, Davis GL, Lopez R, Boone J, et al. Ascitic fluid lactoferrin for diagnosis of spontaneous bacterial peritonitis. Gastroenterology. 2008;135:803–7.

Pinzello G, Virdone R, Lojacono F, Ciambra M, Dardanoni G, Fiorentino G, et al. Is the acidity of ascitic fluid a reliable index in making the presumptive diagnosis of spontaneous bacterial peritonitis? Hepatol Baltim MD. 1986;6:244–7.

Propst T, Propst A, Herold M, Schauer G, Judmaier G, Braunsteiner H, et al. Spontaneous bacterial peritonitis is associated with high levels of interleukin-6 and its secondary mediators in ascitic fluid. Eur J Clin Invest. 1993;23:832–6.

Pruimboom WM, Bac DJ, van Dijk AP, Garrelds IM, Tak CJ, Bonta IL, et al. Levels of soluble intercellular adhesion molecule 1, eicosanoids and cytokines in ascites of patients with liver cirrhosis, peritoneal cancer and spontaneous bacterial peritonitis. Int J Immunopharmacol. 1995;17:375–84.

Rodríguez-Ramos C, Galan F, Díaz F, Elvira J, Martín-Herrera L, Girón-González JA. Expression of proinflammatory cytokines and their inhibitors during the course of spontaneous bacterial peritonitis. Dig Dis Sci. 2001;46:1668–76.

Rogers GB, van der Gast CJ, Bruce KD, Marsh P, Collins JE, Sutton J, et al. Ascitic microbiota composition is correlated with clinical severity in cirrhosis with portal hypertension. PLoS ONE. 2013;8, e74884.

Runyon BA. Low-protein-concentration ascitic fluid is predisposed to spontaneous bacterial peritonitis. Gastroenterology. 1986;91:1343–6.

Runyon BA. Monomicrobial nonneutrocytic bacterascites: a variant of spontaneous bacterial peritonitis. Hepatol Baltim MD. 1990;12(4 Pt 1):710–5.

Runyon BA, AASLD. Introduction to the revised American Association for the Study of Liver Diseases Practice Guideline management of adult patients with ascites due to cirrhosis 2012. Hepatol Baltim MD. 2013;57:1651–3.

Runyon BA, Hoefs JC. Ascitic fluid chemical analysis before, during and after spontaneous bacterial peritonitis. Hepatol Baltim MD. 1985;5:257–9.

Runyon BA, Van Epps DE. Diuresis of cirrhotic ascites increases its opsonic activity and may help prevent spontaneous bacterial peritonitis. Hepatol Baltim MD. 1986;6:396–9.

Runyon BA, Morrissey RL, Hoefs JC, Wyle FA. Opsonic activity of human ascitic fluid: a potentially important protective mechanism against spontaneous bacterial peritonitis. Hepatol Baltim MD. 1985;5:634–7.

Runyon BA, Antillon MR, Montano AA. Effect of diuresis versus therapeutic paracentesis on ascitic fluid opsonic activity and serum complement. Gastroenterology. 1989;97:158–62.

Runyon BA, Antillon MR, McHutchison JG. Diuresis increases ascitic fluid opsonic activity in patients who survive spontaneous bacterial peritonitis. J Hepatol. 1992;14:249–52.

Sanai FM, Bzeizi KI. Systematic review: tuberculous peritonitis – presenting features, diagnostic strategies and treatment. Aliment Pharmacol Ther. 2005;22:685–700.

Scemama-Clergue J, Doutrellot-Philippon C, Metreau JM, Teisseire B, Capron D, Dhumeaux D. Ascitic fluid pH in alcoholic cirrhosis: a reevaluation of its use in the diagnosis of spontaneous bacterial peritonitis. Gut. 1985;26:332–5.

Schuetz P, Albrich W, Mueller B. Procalcitonin for diagnosis of infection and guide to antibiotic decisions: past, present and future. BMC Med. 2011;9:107.

Schwabl P, Bucsics T, Soucek K, Mandorfer M, Bota S, Blacky A, et al. Risk factors for development of spontaneous bacterial peritonitis and subsequent mortality in cirrhotic patients with ascites. Liver Int Off J Int Assoc Study Liver. 2015;35:2121–8.

Simberkoff MS, Moldover NH, Weiss G. Bactericidal and opsonic activity of cirrhotic ascites and nonascitic peritoneal fluid. J Lab Clin Med. 1978;91:831–9.

Singer E, Markó L, Paragas N, Barasch J, Dragun D, Müller DN, et al. Neutrophil gelatinase-associated lipocalin: pathophysiology and clinical applications. Acta Physiol Oxf Engl. 2013;207:663–72.

Solà R, Andreu M, Coll S, Vila MC, Oliver MI, Arroyo V. Spontaneous bacterial peritonitis in cirrhotic patients treated using paracentesis or diuretics: results of a randomized study. Hepatol Baltim MD. 1995;21:340–4.

Soriano G, Guarner C, Teixidó M, Such J, Barrios J, Enríquez J, et al. Selective intestinal decontamination prevents spontaneous bacterial peritonitis. Gastroenterology. 1991;100:477–81.

Soriano G, Castellote J, Alvarez C, Girbau A, Gordillo J, Baliellas C, et al. Secondary bacterial peritonitis in cirrhosis: a retrospective study of clinical and analytical characteristics, diagnosis and management. J Hepatol. 2010;52:39–44.

Soriano G, Esparcia O, Montemayor M, Guarner-Argente C, Pericas R, Torras X, et al. Bacterial DNA in the diagnosis of spontaneous bacterial peritonitis. Aliment Pharmacol Ther. 2011;33:275–84.

Souza MHLP, Cunha FQ, Martinelli ALC. Interleukin 6 concentration in ascitic fluid of cirrhotic patients: relationship with previous episodes of spontaneous bacterial peritonitis. J Gastroenterol. 2003;38:149–52.

Spahr L, Morard I, Hadengue A, Vadas L, Pugin J. Procalcitonin is not an accurate marker of spontaneous bacterial peritonitis in patients with cirrhosis. Hepatogastroenterology. 2001;48:502–5.

Such J, Francés R, Muñoz C, Zapater P, Casellas JA, Cifuentes A, et al. Detection and identification of bacterial DNA in patients with cirrhosis and culture-negative, nonneutrocytic ascites. Hepatol Baltim MD. 2002;36:135–41.

Such J, Hillebrand DJ, Guarner C, Berk L, Zapater P, Westengard J, et al. Nitric oxide in ascitic fluid is an independent predictor of the development of renal impairment in patients with cirrhosis and spontaneous bacterial peritonitis. Eur J Gastroenterol Hepatol. 2004;16:571–7.

Sugihara T, Koda M, Maeda Y, Matono T, Nagahara T, Mandai M, et al. Rapid identification of bacterial species with bacterial DNA microarray in cirrhotic patients with spontaneous bacterial peritonitis. Intern Med Tokyo Jpn. 2009;48:3–10.

Tandon P, Garcia-Tsao G. Renal dysfunction is the most important independent predictor of mortality in cirrhotic patients with spontaneous bacterial peritonitis. Clin Gastroenterol Hepatol Off Clin Pract J Am Gastroenterol Assoc. 2011;9:260–5.

Tao L, Ning H-J, Nie H-M, Guo X-Y, Qin S-Y, Jiang H-X. Diagnostic value of adenosine deaminase in ascites for tuberculosis ascites: a meta-analysis. Diagn Microbiol Infect Dis. 2014;79:102–7.

Terg R, Fassio E, Guevara M, Cartier M, Longo C, Lucero R, et al. Ciprofloxacin in primary prophylaxis of spontaneous bacterial peritonitis: a randomized, placebo-controlled study. J Hepatol. 2008;48:774–9.

Terg R, Casciato P, Garbe C, Cartier M, Stieben T, Mendizabal M, et al. Proton pump inhibitor therapy does not increase the incidence of spontaneous bacterial peritonitis in cirrhosis: a multicenter prospective study. J Hepatol. 2015;62:1056–60.

Titó L, Rimola A, Ginès P, Llach J, Arroyo V, Rodés J. Recurrence of spontaneous bacterial peritonitis in cirrhosis: frequency and predictive factors. Hepatol Baltim MD. 1988;8:27–31.

Vieira SMG, da Silveira TR, Matte U, Kieling CO, Ferreira CT, Taniguchi A, et al. Amplification of bacterial DNA does not distinguish patients with ascitic fluid infection from those colonized by bacteria. J Pediatr Gastroenterol Nutr. 2007;44(5):603–7.

Wiest R, Krag A, Gerbes A. Spontaneous bacterial peritonitis: recent guidelines and beyond. Gut. 2012;61:297–310.

Wright EK, De Cruz P, Gearry R, Day AS, Kamm MA. Fecal biomarkers in the diagnosis and monitoring of Crohn's disease. Inflamm Bowel Dis. 2014;20:1668–77.

Wu SS, Lin OS, Chen YY, Hwang KL, Soon MS, Keeffe EB. Ascitic fluid carcinoembryonic antigen and alkaline phosphatase levels for the differentiation of primary from secondary bacterial peritonitis with intestinal perforation. J Hepatol. 2001;34:215–21.

Zapater P, Francés R, González-Navajas JM, de la Hoz MA, Moreu R, Pascual S, et al. Serum and ascitic fluid bacterial DNA: a new independent prognostic factor in noninfected patients with cirrhosis. Hepatol Baltim MD. 2008;48:1924–31.

Part V

Resources

Recommended Resources on Biomarkers of Liver Diseases

46

Rajkumar Rajendram, Vinood B. Patel, and Victor R. Preedy

Contents

Abstract

There are a variety of diseases that affect the liver ranging from those induced by toxins such as drugs and alcohol to those caused by viruses, poor diets, and the adverse impact of lifestyle factors. In the USA, for example, the prevalence of nonalcoholic fatty liver disease is about 20%, corresponding to about 30 million people. In China there are an estimated 300 million with liver disease. Translated worldwide, such prevalence imposes a considerable global burden. The complex pathogenesis of liver disease creates a plethora of potential biomarkers which can be used at different stages of liver disease, from initiating events to therapeutic treatments. Biomarker discovery is an ongoing process from identification to applications. To aid this dialogue, this chapter identifies a variety of relevant

R. Rajendram (✉)
Diabetes and Nutritional Sciences Research Division, Faculty of Life Science and Medicine, School of Biomedical and Health Sciences, King's College London, London, UK

Department of Internal Medicine, King Abdulaziz Medical City, National Guard Hospital Affairs, Riyadh, Saudi Arabia
e-mail: afmsingh+1@gmail.com; rajkumarrajendram@doctors.org.uk

V.B. Patel
Department of Biomedical Science, Faculty of Science and Technology, University of Westminster, London, UK

V.R. Preedy
Diabetes and Nutritional Sciences Research Division, Faculty of Life Science and Medicine, King's College London, London, UK

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3_46

resources including regulatory and professional bodies, journals on liver disease and biomarkers, books, emerging techniques, and websites relevant to evidence-based biomarker discovery use of biomarkers in diseases.

Keywords
Biomarkers • Diseases of the liver • Evidence • Resources • Books • Journals • Regulatory bodies • Professional societies

Key Points

- The prevalence of liver disease is extremely high.
- Worldwide it has been estimated by other authors that there are 600 million people with nonalcoholic fatty liver disease (NAFLD). There are 150 million worldwide with alcoholic liver disease. Hepatitis C virus (HCV)-specific antibody-positive people is 185 million.
- Biomarkers are an emerging area of significant potential value in hepatology.
- The complex pathogenesis of liver disease creates a plethora of potential biomarkers using a variety of analytical platforms.
- This chapter lists resources on the regulatory bodies, journals, books, professional bodies, and websites that are relevant to an evidence-based approach to the use of biomarkers of diseases of the liver.

Introduction

Liver diseases are increasing and common causes of morbidity and mortality (Aithal et al. 2012; Wang et al. 2014). For example, one hospital study of inpatients in China showed that alcoholic liver disease increased over 20-fold in 10 years and hepatocellular carcinoma increased by just over 25-fold (Wang et al. 2014). The prevalence of nonalcoholic fatty liver disease in the USA is about 20%, corresponding to about 30 million people (Lazo et al. 2013). Worldwide is estimated that there are 600 million with nonalcoholic fatty liver disease (NAFLD) and 150 million with alcoholic liver disease (Wang et al. 2014). Those who are hepatitis C virus (HCV)-specific antibody positive amount to 185 million on a worldwide basis (Wang et al. 2014). As a consequence, liver diseases have a very large impact on the overall global burden of disease. However, in many instances diagnosis is difficult because characteristically symptoms are absent until late in the natural history of the disease. For cirrhosis, the most reliable diagnostic test is liver biopsy, but this is very invasive and a risk factor of adverse events (Aithal et al. 2012). Research on biomarkers is therefore an emerging field of great interest to hepatologists (doctors who specialize

in the management of patients with liver disease). Biomarkers are characteristics measured as indicators of normal biological processes, pathogenic processes, or responses to treatment (Atkinson et al. 2001). Biomarkers are of significant clinical value. Traditionally markers of liver disease were obtained from biofluids such as blood or serum (Aithal et al. 2012). On the other hand genetic, epigenetic, and bioimaging biomarkers can now be used for the diagnosis, prognosis, and epidemiology of diseases of the liver. As with other diseases, there are four main uses for biomarkers in diseases of the liver or indeed any other organ: (i) risk stratification, (ii) diagnosis, (iii) prognostication, and (iv) monitoring response to treatment (Rajendram et al. 2017).

The complex pathogenesis of liver disease, which ranges from steatosis (fatty infiltration), inflammation, and apoptosis to fibrosis, creates a plethora of potential new biomarkers or existing biomarkers that necessitate verification and further development (Aithal et al. 2012). Several putative biomarkers of liver injury have been discovered through omic technologies such as genomics and proteomics (Aithal et al. 2012).

Translating research on novel biomarkers into clinical practice is fraught with difficulty. However, some biomarkers are already in routine use by hepatologists. Examples include physiological biomarkers (e.g., serum bilirubin and albumin) and biomarkers of liver damage (e.g., aspartate transaminase activity) and hormonal biomarkers (e.g., alpha fetoprotein). Very often these are used in concert with the identification and application of novel biomarkers. Biomarkers increase the accuracy of diagnosis and facilitate clinical decision making of the management of liver disease. Of course it is important to point out that there are many more biomarkers other than those mentioned in the aforementioned text and these are described in this book. However, it is incredibly difficult even for those interested in liver disease (experimental scientists and clinicians) to remain up-to-date with the rapid pace of the developments in this field. Determining which available sources are reliable is also problematic. To address this, we have therefore produced tables containing resources. They aim to assist colleagues who are interested in understanding more about biomarkers of liver diseases. The experts who assisted with the compilation of these tables of resources are acknowledged below.

Definitions, measurement and applications of biomarkers can be found in this book and also via the recommended resources in the tables below.

Tables 1–7 list the most up-to-date information on the regulatory bodies (Table 1), professional bodies (Table 2), journals on diseases affecting the liver and biomarkers (Table 3), books on biomarkers (Table 4), books on diseases affecting the liver (Table 5), emerging techniques and platforms (Table 6), and websites (Table 7) that are relevant to an evidence-based use of biomarkers in diseases affecting the liver.

Table 1 Regulatory bodies and organisations. This table lists the regulatory bodies and organisations involved with various aspects of biomarkers (From Rajendram et al. (2017))

American Association Clinical Chemistry (AACC). www.aacc.org/
Biomarkers Consortium. www.biomarkersconsortium.org
Biomarker, Imaging and Quality of Life Studies Funding Program, National Cancer Institute, USA. www.cancer.gov/aboutnci/organization/ccct/funding/BIQSFP
Biomarker Qualification Program US Food and drug administration. http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/ucm284076.htm
Centers for Disease Control and Prevention (CDC). www.cdc.gov/globalhealth/countries/egypt
European Medicines Agency. www.ema.europa.eu/ema/index.jsp?curl=pages/special_topics/general/general_content_000349.jsp
US Food and Drug Administration (FDA). www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/ucm284076.htm
International Federation of Clinical Chemistry and laboratory Medicine (IFCC). www.ifcc.org
Medicines and Healthcare Products Regulatory Agency (MHRA). www.mhra.gov.uk
National Institutes of Health. www.nlm.nih.gov

Table 2 Professional societies. This table lists the professional societies involved with biomarkers and/or diseases affecting the liver

American Association for the Study of Liver Diseases (AASLD)
www.aasld.org
American Cancer Society
http://www.cancer.org/
Asociación Latinoamericana para el Estudio del Higado
alehlatam.org
Associação Brasileira de Transplante de Órgãos
www.abto.org.br/abtov03
European Association for the Study of the Liver (EASL)
www.easl.eu
Galilee Medical Center
www.gmc.org.il
Sociedade Brasileira de Hepatologia
www.sbhepatologia.org.br

Table 3 Journals publishing on diseases affecting the liver and biomarkers. This table lists the top 25 journals publishing original research and review articles related to diseases affecting the liver. The list was generated from SCOPUS (www.scopus.com) using general descriptors. The journals are listed in descending order of the total number of articles published in the past 5 years. Of course, different indexing terms or different databases will produce different lists so this is a general guide only. For example, journals associated with biomarker discovery will produce a different list

Plos One
World Journal of Gastroenterology
Hepatology

(*continued*)

Table 3 (continued)

Liver International
Journal of Hepatology
Liver Transplantation
European Journal of Gastroenterology and Hepatology
Annals of Hepatology
Hepato Gastroenterology
Journal of Gastroenterology and Hepatology Australia
Hepatology Research
Transplantation Proceedings
Digestive Diseases and Sciences
Scientific Reports
Gastroenterology
Tumor Biology
Journal of Surgical Research
Transplantation
Zhonghua Gan Zang Bing Za Zhi Zhonghua Ganzangbing Zazhi Chinese Journal of Hepatology
Hepatobiliary and Pancreatic Diseases International
Toxicological Sciences
Oncotarget
World Journal of Hepatology
Alimentary Pharmacology and Therapeutics
International Journal of Molecular Sciences

Table 4 Relevant books on biomarkers. This table lists books on biomarkers

Analysis of Biomarker Data: A Practical Guide. Looney SW, Hagan JL. Wiley, 2015, USA
Aptamer Handbook: Functional Oligonucleotides and Their Applications. Klussmann S (editor). Wiley-VCH, 2006, Weinheim
Aptamers in Bioanalysis. Mascini M. Wiley-Interscience, 2009, USA
Biomarker Guide. Peters KE, Walters CC, Moldowan JM. Cambridge University Press, 2010, USA
Biomarkers: In Medicine, Drug Discovery, and Environmental Health. Vaidya VS, Bonventre JV. John Wiley & Sons, 2010, USA
Biomarkers of Disease: An Evidence-Based Approach. Trull AK, Demers LM, Holt DW, Johnston A, Tredger JM, Price CP, Cambridge University Press, 2002, UK
Biomarkers in Oncology: Prediction and Prognosis. H-J Lenz, Springer, 2013, Germany
Development and Application of Biomarkers. Lundblad RL, CRC Press, 2016, USA
Handbook of Biomarkers. Kewal KJ. Lippincott, 2010, USA
The Path from Biomarker Discovery to Regulatory Qualification. Goodsaid F, Mattes W. Academic Press, 2013, USA

Table 5 Relevant books on diseases affecting the liver. This table lists books on diseases affecting the liver

Fructose: function and health implications. Assy N, Food Science and Technology, Israel
Handbook of Liver Disease, 3rd Edition. Friedman LS, Keeffe EB. Saunders, 2012, USA
Mechanism of Fatty Liver and CAD. Assy N, in Tech, 2011, Israel
Molecular Pathology of Liver Diseases. Monga SPS. Springer, 2011, USA
Olives and Olive Oil in Health and Disease. Assy N, Oxford Academic Press, 2010, Israel
Tratado de hepatologia. Mattos AA, Dantas-Corrêa EB, Rubio, 2010, Brazil

Table 6 Sources and resources for emerging techniques and platforms. This table lists some emerging sources, resources platforms in biomarker discovery, and application which are relevant to diseases of the liver

Biobanking and Biomolecular Resources Research Infrastructure
bbmri.eu
Fibroscan
www.echosens.com
University of Zurich Progenetix database
progenetix.org/cgi-bin/pgHome.cgi
FLUIDIGM CyTOF2
www.fluidigm.com/products/helios
Quanterix
www.quanterix.com/products/simoa-hd-1-analyzer

Table 7 Relevant Internet resources. This table lists some Internet resources on biomarkers and diseases affecting the liver

Bar-ilan University Faculty of Medicine, Galilee
Medicine.biu.ac.il
Biomarkers Test (BMT)
www.biomarkers.it
Biomed Central (BMC) Biomarkers
biomarkerres.org
EBSCO Health
health.ebsco.com
Medscape
www.medscape.com
News Medical
www.news-medical.net/health/What-is-a-Biomarker.aspx

References

Aithal GP, Guha N, Fallowfield J, Castera L, Jackson AP. Biomarkers in liver disease: emerging methods and potential applications. Int J Hepatol. 2012. www.hindawi.com/journals/ijh/2012/437508/. Accessed 18 Sept 2016.

Atkinson AJ, Colburn WA, DeGruttola VG, DeMets DL, Downing GJ, Hoth DF, Oates JA, Peck CC, Schooley RT, Spilker BA, Woodcock J, Zeger SL, NCI-FDA Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001;69:89–95.

Lazo M, Hernaez R, Eberhardt MS, Bonekamp S, Kamel I, Guallar E, Koteish A, Brancati FL, Clark JM. Prevalence of nonalcoholic fatty liver disease in the United States: the third national health and nutrition examination survey, 1988–1994. Am J Epidemiol. 2013;178(1):38–45.

Rajendram R, Patel VB, Preedy VR. Recommended resources on biomarkers of bone diseases. In: Patel VB, Preedy VR, editors. Biomarkers in bone disease. Springer; 2017. In press.

Wang F-S, Fan J-G, Zhang Z, Gao B, Wang H-Y. The global burden of liver disease: the major impact of China. Hepatology. 2014;60(6):2099.

Index

V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Liver Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7675-3