Kewal K. Jain

The Handbook of Biomarkers

Second Edition

Humana Press

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Preface to the Second Edition

Tremendous advances have taken place since the publication of the first edition of this book in 2010, both in the discovery as well as applications of biomarkers. This has necessitated the preparation of a second edition to provide up-to-date information on this topic. Most of the original book has been rewritten, and new chapters are added. Biomarkers are involved in every therapeutic area, particularly cancer. Pharmaceutical companies are using biomarkers for drug discovery and development, particularly in clinical trials. Biomarkers are now closely linked to practice of personalized medicine.

Uniform style of presentation, characteristic of a single-author book, has been maintained with minimal overlap between chapters. Instead of a bibliography at the end of the book, the references cited in each chapter are appended at the end of the chapter. This will be convenient for specialists such as oncologists, neurologists, cardiologists, etc., who may wish to study only the chapters relevant to their area of interest. Nevertheless, the first few and the last three chapters are of general interest for a wide range of readers including basic scientists, pharmacologists, general physicians, and those working in clinical laboratories and the pharmaceutical industry.

As in the first edition, the author acknowledges the help and encouragement from the Springer editorial staff and management, particularly David Casey and Patrick Marton.

Basel, Switzerland — Kewal K. Jain, MD

Preface to the First Edition

This book is an overview of the state-of-art of biomarkers. A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of a physiological as well as a pathological process or response to a therapeutic intervention. Although there is nothing new about biomarkers such as glucose for diabetes and blood pressure for hypertension, the current focus is on molecular biomarkers, which have taken the center stage in the development of molecular medicine. Molecular diagnostic technologies have enabled the discovery of molecular biomarkers, and are helping in the definition of their role in pathomechanism of disease. Biomarkers form the basis of development of diagnostic assays as well targets for drug discovery. Effect of drugs, in clinical trials as well as in practice, can be monitored by biomarker assays.

There is a tremendous amount of literature on biomarkers, but there is no comprehensive source of information on the topic. Of the thousands of biomarkers that are being discovered, relatively few are being validated for further applications, and it is difficult to evaluate the potential of a biomarker. This book describes different types of biomarkers and their discovery using various "-omics" technologies such as proteomics and metabolomics along with the background information for evaluations of biomarkers as well as the procedures for their validation and use in clinical trials. Biomarkers are first described according to technologies and then according to various diseases. An important feature is correlation with diseases and classification of biomarkers, which provides the reader with a guide to sort out current and future biomarkers.

This book would be an important source of information on biomarkers for scientists as well as physicians, and those involved in drug discovery and development. Many of the regulatory issues concerning biomarkers are related to proteomics, molecular diagnostics, and pharmacogenomics/pharmacogenetics. By facilitating the combination of therapeutics with diagnostics, biomarkers will play an important role in the development of personalized medicine, which is an important emerging trend in healthcare.

Basel, Switzerland — Kewal K. Jain, MD

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About the Author

Kewal K. Jain is a neurologist/neurosurgeon by training and has been working in the biotechnology/biopharmaceuticals industry for several years. He received graduate training in both Europe and USA, has held academic positions in several countries and is a Fellow of the Faculty of Pharmaceutical Medicine of the Royal College of Physicians of UK. Currently he is a consultant at Jain PharmaBiotech. Professor Jain's 470 publications include 28 books (5 as editor+ 23 as author) and 50 special reports, which have covered important areas in biotechnology, gene therapy and biopharmaceuticals. The following Jain PharmaBiotech reports are relevant to biomarkers: proteomics, molecular diagnostics, nanobiotechnology, and personalized medicine. Recent books include: *Handbook of Nanomedicine* (Springer 2008, Chinese edition by Peking University Press 2011, 3rd ed 2017), *Textbook of Personalized Medicine* (Springer 2009; Japanese ed 2012; 2nd ed Springer 2015), *Handbook of Biomarkers* 1st ed. (Springer 2010; Chinese ed, Chemical Industry Press, 2016), *Handbook of Neuroprotection* (Springer 2011), *Applications of Biotechnology in Cardiovascular Therapeutics* (Springer 2011), *Applications of Biotechnology in Neurology* (Springer 2013), *Applications of Biotechnology in Oncology* (Springer 2014), and *Textbook of Hyperbaric Medicine*, 6th ed. (Springer 2017). He has also edited *Applied Neurogenomics* (Springer 2015).

Abbreviations

2D GE	2-Dimensional gel electrophoresis
AD	Alzheimer's disease
BNP	B-type natriuretic peptide
CHD	Coronary heart disease
CHF	Congestive heart failure
CNS	Central nervous system
CO	Carbon monoxide
CRADA	Cooperative research and development agreement (between a US federal laboratory and one or more non-federal parties)
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CT	Computer tomography
CTC	Circulating tumor cell
DT-MRI	Diffusion-tensor MRI
EGFR	Epithelial growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tags
FDA	Food and Drug Administration, USA
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescent in situ hybridization
fMRI	Functional magnetic resonance imaging
GC	Gas chromatography
GFAP	Glial fibrillary acidic protein
GWAS	Genome-wide association study
H_2S	Hydrogen sulfide
Hs-CRP	High sensitivity C-reactive protein
IHC	Immunohistochemistry
IL	Interleukin
KRAS	Kirsten rat sarcoma viral oncogene homolog
LC	Liquid chromatography
LCM	Laser capture microdissection

LDH	Lactic dehydrogenase
LDT	Laboratory developed test
Lp-PLA2	Lipoprotein-associated phospholipase A2
MALDI	Matrix-assisted laser desorption/ionization
MALDI-MS	Matrix-assisted laser desorption mass spectrometry
MCP-1	Monocyte chemoattractant protein-1
miRNA	MicroRNA
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MS	Mass spectrometry
mtDNA	Mitochondrial DNA
NCI	National Cancer Institute
NIH	National Institutes of Health, USA
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
NYHA	New York Heart Association
PCR	Polymerase chain reaction
PET	Positron emission tomography
PKC	Protein kinase C
POC	Point-of-care
PPAR	Peroxisome proliferator-activator receptor
PSA	Prostate-specific antigen
PSMA	Prostate-specific membrane antigen
RAS	Rat sarcoma viral oncogene homolog
RCAT	Rolling circle amplification technology
RNAi	RNA interference
SELDI-TOF	Surface-enhanced laser desorption and ionization-time of flight
sICAM-1	Soluble intercellular adhesion molecule-1
SNP	Single nucleotide polymorphisms
SPR	Surface plasma resonance
TIMI	Thrombolysis in myocardial infarction
USPTO	United States Patent & Trademark Office

Chapter 1
Introduction

Definitions

There are several definitions of biomarkers. A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of a physiological as well as a pathological process or pharmacological response to a therapeutic intervention. Classical biomarkers are measurable alterations in blood pressure, blood lactate levels following exercise and blood glucose in diabetes mellitus. Any specific molecular alteration of a cell on DNA, RNA, metabolite or protein level can be referred to as a molecular biomarker. In the era of molecular biology, biomarkers usually mean molecular biomarkers and can be divided into three broad categories:

1. Those that track disease progression over time and correlate with known clinical measures
2. Those that detect the effect of a drug
3. Those that serve as surrogate endpoints in clinical trials

While researchers are studying all three categories, biotechnology and pharmaceutical companies favor using biomarkers as drug discovery tools – not only to detect biological responses to experimental drugs but also to aid in the discovery of new targets for therapeutic intervention. A biomarker can be as simple as a laboratory test or as complex as a pattern of genes or proteins. From a practical point of view, the biomarker would specifically and sensitively reflect a disease state and could be used for diagnosis as well as for disease monitoring during and following therapy. The term "negative biomarker" is used for a marker that is deficient or absent in a disease.

Surrogate endpoint is a biomarker that is intended to serve as a substitute for a clinically meaningful endpoint and is expected to predict the effect of a therapeutic intervention. A clinical endpoint is a clinically meaningful measure of how a patient feels functions or survives. Clinical endpoints may be further classified as intermediate endpoints, which are clinical endpoints that are not the ultimate outcome, but are nonetheless of real clinical usefulness, e.g. exacerbation rate, and ultimate clinical

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outcomes, which are clinical endpoints reflective of the accumulation of irreversible morbidity and survival. These definitions indicate a clear hierarchical distinction between biomarkers and surrogate endpoints. While numerous laboratory biomarkers may be associated with a particular disease state, the term 'surrogate' indicates the ability of a biomarker to provide information about the clinical prognosis or efficacy of a therapy. The word 'surrogate' implies a strong correlation with a clinical endpoint, but in order to be clinically useful a surrogate must provide information about prognosis or therapeutic efficacy in a significantly shorter time than would be needed by following the clinical endpoint.

Historically, successful surrogates have linked effects on biomarkers for single effects in large populations but this framework needs to be expanded because it does not recognize multidimensional quality of clinical response and thus conflicts with current goals for individualized therapy. There is also the need to include possibility that multiple biomarkers may provide useful information in aggregate. A biomarker is valid if:

1. It can be measured in a test system with well established performance characteristics
2. Evidence for its clinical significance has been established

Historical Aspects of Biomarkers

Historical landmarks in discovery and development of biomarkers are shown in Table 1.1.

Classification of Biomarkers

A classification of biomarkers is shown in Table 1.2.

Biomarker as a Response to Therapeutic Intervention

A biomarker can be a pharmacologic response to therapeutic intervention. A pharmacogenetic test is an assay intended to study interindividual variations in DNA sequence related to drug absorption and disposition (pharmacokinetics) or drug action (pharmacodynamics), including polymorphic variation in the genes that encode the functions of transporters, metabolizing enzymes, receptors, and other proteins.

A pharmacogenomic test is an assay intended to study interindividual variations in whole-genome or candidate gene, SNPs, haplotype markers, or alterations in

Table 1.1 Historical landmarks in discovery and development of biomarkers

Year	Landmark
1847	The first laboratory test for a protein cancer biomarker, the Bence Jones protein in urine.
1954	Test for the measurement of transaminases in myocardial infarction (Karmen et al. 1954).
1960s	The term "biomarker" started to appear in the literature on metabolites and biochemical abnormalities associated with several diseases.
1967	An improved test for myocardial infarction based on a biomarker – serum creatine phosphokinase (Rosalki 1967).
1971	Report of carcinoembryonic antigen (CEA) as biomarker of cancer (Moore et al. 1971).
1987	Troponin I as biomarker for myocardial infarction (Cummins et al. 1987)
Early 1990s	Accelerator mass spectrometry used for analysis of biological samples for biomarkers
1995	Applications of proteomics for discovery of biomarkers and use in molecular diagnostics.
1999	Emergence of metabolomics for study of biomarkers.
2000	Sequencing of the human genome completed, opening the way for discovery of gene biomarkers.
2005	Discovery and application of biomarkers becomes a major activity in biotechnology and biopharmaceutical industries.

gene expression or inactivation that may be correlated with pharmacological function and therapeutic response. In some cases, the pattern or profile of change is the relevant biomarker, rather than changes in individual markers.

Biomarkers in relation to therapeutic response can be dynamic or static. Dynamic biomarkers describe disease progression and associated treatment response, and are widely used in patient care and drug development, e.g. dynamics of prostate specific antigen for response to prostate cancer treatment (van Hasselt et al. 2015). Static biomarkers are prognostic and predict a clinical response e.g. gene expression signatures for prediction of clinical benefit of anticancer drugs (Cardoso et al. 2016).

Pharmacokinetic/Pharmacodynamics Biomarkers

Mechanism-based pharmacokinetic/pharmacodynamic (PK/PD) models differ from empirical descriptive models in that they contain specific expressions to characterize processes on the causal path between drug administration and effect. Mechanism-based PK/PD models have much improved properties for extrapolation and prediction. As such, they constitute a scientific basis for rational drug discovery and development. Within the context of mechanism-based PK/PD modeling, a biomarker is defined as a measure that characterizes, in a strictly quantitative manner, a process,

Table 1.2 Classification of biomarkers

Disease biomarkers: type 0
Clues to pathomechanism of a disease
Diagnostic biomarkers for early detection of disease
Global biomarkers for tracking disease progression over time, e.g. tumor size
Process biomarkers are specific lab measures to capture molecular aspect of pathogenesis of the disease
Prognostic biomarker for prognosis or outcome of disease
Progression biomarkers have predictive power for a desired outcome and can serve as surrogate biomarkers
Diagnostic biomarkers
Molecular diagnostics, e.g. CA-125 for ovarian cancer
Biomarkers as links between diagnostics and therapeutics
Pattern diagnosis, e.g. serum protein biomarker pattern diagnosis of ovarian cancer
Biomarkers for drug discovery
Target biomarker: reports interaction of the drug with its target
Disease biomarkers as targets for drug discovery
Predictive biomarkers
Biomarker associated with a risk for disease as a candidate for a screening test
To predict disease at presymptomatic stage: autoantibodies
To predict the effect of a drug on disease
To predict the toxicity of a drug
Biomarkers to detect drug effects: type I biomarkers
Efficacy biomarker: indicator of beneficial effect of a drug
Mechanism biomarker: reports a downstream effect of a drug
Pharmacodynamic biomarker: indicator of pharmacological mechanisms and response to a therapy
Toxicity biomarker: reports toxicological effect of a drug in in vitro or in vivo systems
Translation biomarker
A biomarker that can be applied in both a preclinical and clinical setting
Biomarkers as surrogate endpoints in clinical trials: type II biomarkers
As a substitute measure for clinical outcome, e.g. cholesterol levels in statin therapy
In vivo imaging as endpoint: MRI of multiple sclerosis lesions in interferon therapy
Valid biomarkers: validated in clinical trials

which is on the causal path between drug administration and effect. Biomarkers relevant to PK/PD are:

1. Genotype/phenotype for determining drug response
2. As indicatator of concentration of a drug or drug metabolite in the body
3. As indicators of molecular target activation or occupancy by a drug
4. Physiological measures
5. Pathophysiological measures

Predictive Biomarkers

Biomarkers may be used to predict the efficacy or toxicity of a drug. Finding reliable biomarkers that are indicators of a certain response is difficult. So when looking for biomarkers that can predict a certain clinical outcome the task becomes even more challenging. Biomarkers for predicting toxicity, which is often dose-related, are difficult. These effects are usually studied by increasing the dose of a compound until toxicity is observed. However, the predictive value of such an approach in patients is very limited. What is needed is a biomarker that will predict toxicity in a certain patient population.

In the chemoinformatics approach, chemistry-related toxicity can be predicted with the help of databases of known drugs that links phenotypic toxicity to a specific characteristic of a compound. However, other approaches are required for determining genomic-based toxicity.

Biomarkers are used in toxicogenomics as well. Toxicogenomics is based on the idea that if the environment inside a cell is altered by an external stimulus, some of the cell's genes will likely express themselves in an atypical way. The more toxic the external stimulus, the greater the number of genes that will be altered. Conversely, if the stimulus is benign, then very few genes will change. Predictive toxicogenomics, i.e. the acquisition of advanced knowledge of the safety profile of a compound using genomic biomarkers, is a technology that provides much optimism for improving early drug discovery decisions. Toxicogenomics creates an opportunity to shift attrition to earlier stages in drug development to a point where course-corrective action can be taken with relatively lower financial costs, thus improving the efficiency of the drug development process. Toxicogenomics can be used for predicting toxicity, both in vivo and in vitro, by using classification algorithms and toxicogenomic databases for biomarker discovery and validation.

Valid Biomarkers

A valid biomarker is defined as a biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is an established scientific framework or body of evidence that elucidates the physiologic, toxicologic, pharmacologic, or clinical significance of test results. Validation of a biomarker is context-specific and the criteria for validation will vary with the intended use of the biomarker. The clinical utility (e.g. predict toxicity, effectiveness or dosing) and use of epidemiology/ population data (e.g. strength of genotype-phenotype associations) are examples of approaches that can be used to determine the the necessary criteria for validation. Factors to be considering for approaching biomarker validation are:

1. Consistency of the biomarker with known or expected physiologic or pathophysiologic effects on the process
2. Acceptance and adoption by experts in the field

Table 1.3 Terminology of clinically relevant biomarkers of disease

Term	Application
Predisposition biomarker	To identify predisposition to a disease, e.g. genetic
Screening biomarkers	To identify those suffering from a disease
Staging biomarker	To determine the stage of progression of the disease
Prediction biomarker	To predict the course of the disease
Prognostic biomarker	To assess disease progression and outcome.
Recurrence monitoring biomarkers	To identify recurrence of the disease

3. Utilization by pharmaceutical companies for decision-making about advancing drugs into further development.

Table 1.3 lists the terms used for disease biomarkers in clinical development, which is an expansion of type 0 biomarkers listed in Table 1.2. Regulatory aspects of biomarker validation will be discussed in Chap. 19.

Types of Biomarkers

There are many ways of classifying biomarkers as reflected in the rest of this report. The biomarkers may be simple molecules such as metabolites, carbohydrates (e.g. glucose), steroids and lipids. Less simple are peptides and proteins such as insulin, hemoglobin A and C, prostate specific antigen and C-reactive protein. More complex biomarkers are cells such as platelets or T cells and autoantibodies. Patients as clinical phenotypes are most complex but this topic will not be discussed in this report.

Genes as Biomarkers

A gene is a sequence of chromosomal DNA that is required for the production of a functional protein or a functional RNA molecule. Genes range in size from small (1.5 kb for globin gene) to large (~2000 kb for Duchenne muscular dystrophy gene). A gene includes not only the actual coding sequences but also adjacent nucleotide sequences required for the proper expression of genes, i.e., for the production of a normal mRNA molecule. Mature mRNA is about one-tenth the size of the gene from which it is transcribed. The same DNA strand of a gene is always translated into mRNA so that only one kind of mRNA is made for each gene. Transcription is gene in action. Genes are often described as blueprints of life ands transmit inherited traits from one generation to another.

The activity of a gene, so called gene "expression" means that its DNA is used as a blueprint to produce a specific protein. Not all the genes are expressed in a typical human cell and those that are expressed vary from one cell to another. Patterns in which a gene is expressed provide clues to its biological role. Malfunctioning of genes is involved in most diseases, not only inherited ones. All functions of cells, tissues and organs are controlled by differential gene expression. As an example, red blood cells contain large amounts of the hemoglobin protein that is responsible for carrying oxygen throughout the body. The abundance of hemoglobin in red blood cells reflects the fact that its encoding gene, the hemoglobin gene, is actively transcribed in the precursor cells that eventually produce red blood cells. In all other cells of the body, the hemoglobin gene is silent. Accordingly, hemoglobin is present only in red blood cells. It is now well established that differential gene expression results in the carefully controlled (or regulated) expression of functional proteins, such as hemoglobin and insulin.

Silent Gene Mutations

So-called silent, or synonymous, mutations change the sequence of a gene but not the protein it encodes. Silent mutations can occur in noncoding regions (outside of genes or within introns), or they may occur within exons. When they occur within exons they either do not result in a change to the amino acid sequence of a protein (synonymous substitution) or result in the insertion of an alternative amino acid with similar properties to that of the original amino acid; in either case there is no significant change in phenotype. Although previously thought to have no biological effect, silent mutations are now considered to be associated with disease states. Identification of silent mutations as biomarkers may not only improve diagnostics, but enable gene-based therapies as well, by giving the patient an improved version of the gene.

Epigenetic Biomarkers

Epigenetics is the study of chemical modifications in DNA and histones that regulate the gene expression or cellular phenotype. During the past decade this term has evolved further with elucidation of various mechanisms involved in regulating gene expression. Technologies for analyzing epigenetic changes, e.g. methylated DNA, microRNA (miRNA) expression, post-translational modifications on histones, have disclosed new biomarkers for the study of neurodegenerative diseases, multifactorial complex diseases, rare diseases and cancer (García-Giménez et al. 2012).

Lifestyle, stress, drugs, physiopathological situations and pharmacological interventions have a great impact on the epigenetic code of the cells by altering the methylome, miRNA expression and the covalent histone modifications. Since there is a need to find new biomarkers and improve diagnosis for several diseases, research

on epigenetic biomarkers for molecular diagnostics encourages the translation of this field from the bench to clinical practice. In this context, deciphering intricate epigenetic modifications involved in several molecular processes is a challenge that remains to be addressed. Several high-throughput technologies and laboratory techniques are available for epigenetic studies that can be reliably used in a clinical diagnostic laboratory (Sandoval et al. 2013). Most promising advances in epigenetic biomarkers have been achieved in cancer (see Chap. 13). Several autoimmune disorders, diseases of the nervous system, and rare syndromes have specific impaired molecular pathways with involvement of epigenetic mechanisms, thus providing opportunities for the discovery of new epigenetic biomarkers. It is anticipated that epigenome-wide association studies will increasingly complement genome-wide association studies in the study of novel disease genes and clinically relevant biomarkers, along with use of bioinformatics.

Proteins as Biomarkers

Proteins are fairly large molecules, made up of strings of amino acids linked like a chain. There are 20 amino acids, and proteins range in length from a few to over a thousand amino acids. Different combinations of amino acids link to form tens of thousands of proteins. Proteins usually contain thousands of atoms precisely arranged in a 3-D structure that is unique for each type.

As a protein is made, it "folds" itself into a complex, 3D shape, like a piece of ribbon that has been crumpled up. Each protein has one folded shape, and consistently folds into it, usually in less than a second. That complicated folded shape dictates how the protein works, and also how it interacts with other entities.

The specific sequence of amino acids that make up each protein is coded by a gene in the DNA of living cells. A protein cannot be synthesized without its mRNA being present, but a protein can persist in the cell when its mRNA is no longer present. However, mRNA may be present in abundance but the message is not translated into proteins. There is, thus, no good correlation between mRNA and protein in a cell at any given time. Protein synthesis is a very complicated process. Ribosomes are the cell's protein factories. RNA bridges in the ribosomes are not just support structures but also a part of the protein forming machinery.

Peptides are small proteins that play a central role in almost all biological processes. They function as biochemical messengers (for example: insulin, calcitonin and angiotensin) or occur as metabolites of proteins.

Proteomics

The term 'proteomics' indicates PROTEins expressed by a genOME and is the systematic analysis of protein profiles of tissues. The term "proteome" refers to all proteins produced by a species, much as the genome is the entire set of genes.

Unlike the genome, the proteome varies with time and is defined as "the proteins present in one sample (tissue, organism, cell culture) at a certain point in time". Proteomics parallels the related field of genomics. Now that the human genome has been sequenced, we face the greater challenge of making use of this information for improving healthcare and discovering new drugs. There is an increasing interest in proteomics technologies now because DNA sequence information provides only a static snapshot of the various ways in which the cell might use its proteins whereas the life of the cell is a dynamic process. In addition to proteins, peptides (low molecular weight proteins) are also biomarkers of disease in body tissues and can be detected by proteomic technologies.

DNA Biomarkers

Genetic information is contained in the cells in the form of DNA. DNA consists of two strands, which resemble a ladder coiled into a spiral shape - the double helix. It is a macromolecule composed of linear array of nucleotides, each of which comprises a base plus a pentose sugar and phosphate. Only four nucleotide bases are normally found in DNA: cytosine (C), thymine (T), adenine (A) and guanine (G). The information content of the DNA is embodied in the sequential arrangement of nucleotides. The assembly of higher order structures comprising multiple proteins bound at distinct DNA sites initiates readout of information encoded in the DNA. DNA contains the instructions for making proteins. There is a need to assess DNA damage because of impact that different insults on genetic material may have on human health.

Mitochondrial DNA

While autosomal nuclear DNA genes are confined to the nucleus, limited to two copies per cell, the mitochondrial DNA (mtDNA) genes are distributed throughout the cytoplasm and are present in numerous copies per cell. The mtDNA molecule is relatively small containing 16,569 nucleotide pairs. Mitochondria are descendent of a "bacterium-like" organism, which had a working relationship with our ancestral cells so that they could produce energy from glucose and oxygen and store this energy in the form of high-energy phosphate bonds of adenosine triphosphate (ATP). As a remnant of its past life, each mitochondrion contains a "private" set of genes that possess the genetic blueprint for the production of proteins and other molecules that are critical to the process of cellular energy production. mtDNA encodes for proteins that are components of the mitochondrial respiratory chain and oxidative phosphorylation system. Mitochondria have a degree of autonomy within the cell by virtue of having their own genome but it is limited because replication and transcription of mtDNA is dependent on nuclear factors such as mitochondrial

transcription factor a. mtDNA differs from DNA in cell nucleus in the following important respects:

- It is strictly maternally inherited, does not recombine and therefore accumulates mutations sequentially.
- It contains few non-coding sequences
- It has a slightly different genetic code, for example, the uridine - guanine - adenine (UGA) codon is read as “tryptophan” rather than a “stop”.

Mitochondrial Mutations

There is growing evidence that defects of mtDNA causes disease. Majority of these defects are due to point mutations or rearrangements of the mitochondrial genome, while others, such as mtDNA deletions, are autosomally-linked. More than 100 mutations of mtDNA been associated with a striking variety of multisystemic as well as tissue-specific human diseases. Disorders due to mutations in genes affecting mitochondrial protein synthesis may erode the bioenergetic capacity of the tissues contributing to the senescence process in aging. In contrast to the remarkable progress in our understanding of etiology, pathogenesis is only partially explained by the rules of mitochondrial genetics and remains largely unclear.

RNA Biomarkers

Ribonucleic acid (RNA) is the other major nucleic acid besides DNA but unlike DNA, it is single-stranded. It contains ribose instead of deoxyribose as its sugar-phosphate backbone, and that uracil (U) instead of thymine (T) in its pyramidine bases. Like DNA, it can be assembled from nucleotides using DNA sequence as a template and RNA polymerase. The structure of an RNA molecule is also determined by its DNA-derived sequence. If proteins are the hardware, RNA is the software controlling how the genes are expressed to make proteins. RNA is unique in being able to store and transmit information as well as process that information.

Classically RNAs can be classified into messenger RNAs (mRNAs), which are translated into proteins, and non-protein-coding RNAs (ncRNAs). mRNA is the short-lived intermediary in the transfer of genetic information from DNA to protein. mRNA is transported out of the nucleus and is translated into protein on the cytoplasmic ribosomes. Transcriptome is the complete set of mRNA molecules of a cell, tissue or an organism. Transcription preserves the whole information content of the DNA sequence that it has been transcribed from, since the RNA has the same base-pairing characteristics.

ncRNA genes produce functional RNA molecules rather than encoding proteins and include transfer RNAs (tRNAs) and ribosomal RNAs (rRNA). rRNAs are highly structured and conserved molecules found in all living organisms and are

well established as phylogenetic markers. ncRNAs are very stable molecules, which represents a very important prerequisite for an excellent biomarker. During the last two decades several ncRNAs have emerged, having a diverse range of functions, from structural through regulatory to catalytic. A dominating category is that of small nucleolar (sno) RNAs, which act as guides to direct pseudouridylation and 2′-O-ribose methylation in rRNA. Other categories are microRNAs (miRNAs), antisense transcripts and transcriptional units containing a high density of stop codons and lacking any extensive open reading frame. tRNAs are the most promising biomarkers as they are present in circulating biological fluids and can be consequently tested with noninvasive and rather inexpensive methodology.

Human mRNAs are present in saliva and can be used as biomarkers of oral cancer, e.g., squamous cell carcinoma. Saliva harbors both full-length and partially degraded forms of mRNA. RNA enters the oral cavity from different sources, and association with macromolecules may protect salivary RNA from degradation. However RNA is unstable and the degradation process is likely to start before the cells are shed from the tissue, limiting its value as a biomarker. The results of measurements of transcript levels in biopsies of oral tissue need to be interpreted with caution. To address the problem of RNA instability, RNA is immediately stabilized after the blood draw by PAXgene (PreAnalytiX). Total RNA is then extracted from PAXgene-stabilized blood and subjected to microarray analysis (Debey-Pascher et al. 2009). Combining RNA stabilization of peripheral blood with bead-based oligonucleotide microarray technology is not only applicable to small single-center studies with optimized infrastructure but also to large scale multicenter trials that are mandatory for the development of predictive biomarkers for disease and treatment outcome.

Transcriptomics

The focus of decoding genomic information for drug discovery has been mostly on proteomics and mRNA (cDNA) analysis. A limitation of this approach is that the information contained within the genome is first expressed in the form of “primary transcripts” before it is processed into mRNA and proteins. The primary transcripts may not lead to the formation of mRNA and proteins but perform crucial cellular functions directly. Transcriptomics is the study of the entire set of RNA transcripts of an organism.

A shared goal in transcript and proteomic profiling is the development of biomarkers and signatures of chemical toxicity. Toxicity profiling with DNA microarrays to measure all mRNA transcripts, or by global separation and identification of proteins, has led to the discovery of better descriptors of toxicity, toxicant classification and exposure monitoring than current indicators. Biomarkers and signature profiles are described for specific chemical toxicants that affect target organs such as liver, kidney, neural tissues, gastrointestinal tract and skeletal muscle, for specific disease models such as cancer and inflammation, and for unique chemical-protein adducts underlying cell injury. The introduction of toxicogenomics databases

supports researchers in sharing, analyzing, visualizing and mining expression data, assist the integration of transcriptomics, proteomics and toxicology datasets, and eventually will enable in silico biomarker and signature pattern discovery.

MicroRNAs

MicroRNAs (miRNAs), small mostly ncRNA gene products, are molecules derived from larger segments of "precursor" RNA that are found in all diverse multicellular organisms. miRNAs are 21–25-nucleotide transcripts that repress gene function through interactions with target mRNAs. miRNAs target the control of gene activity at multiple levels, specifically transcription, translation and protein degradation, i.e. miRNAs act as meta-regulators of expression control. miRNA-mediated gene regulation is guided by the base-pairing rules of Watson and Crick.

Each miRNA is thought to regulate multiple genes, and since >1000 miRNA genes have been identified in humans, the potential regulatory circuitry afforded by miRNA is enormous. Recent studies of miRNA expression implicate miRNAs in viral disease, neurodevelopment, and cancer. In higher eukaryotes, the role of miRNAs in regulating gene expression could be as important as that of transcription factors.

Metabolomics

The human metabolome is best understood by analogy to the human genome, i.e. where the human genome is the set of all genes in a human, the human metabolome is the set of all metabolites in a human. In a systems biology approach, metabolomics provides a functional readout of changes determined by genetic blueprint, regulation, protein abundance and modification, and environmental influence. Metabolomics is the study of the small molecules, or metabolites, contained in a human cell, tissue or organ (including fluids) and involved in primary and intermediary metabolism. It analyzes large arrays of metabolites, thereby extracting biochemical information that reflects the true functional endpoints of biological events. By definition, the metabolome should exclude enzymes, genetic material and structural molecules such as glycosaminoglycans, and other polymeric units that are degraded to small molecules but do not otherwise participate in metabolic reactions.

A related term, metabonomics is the use of NMR technology to study metabolomics, which is defined as the study of metabolic changes. It encompasses metabolomics, metabolite target analysis, metabolite profiling, metabolic fingerprinting, metabolic profiling, and metabonomics. Examination of a sample using multiple mass spectrometry (MS)-based technologies, nuclear magnetic resonance (NMR), integration the data and analysis by proprietary software and algorithms enables faster and more accurate understanding of a disease than previously possible.

The Human Metabolome Database (HMDB) of the Metabolomics Society (http://metabolomicssociety.org/resources/metabolomics-databases) is a freely available electronic database containing detailed information about small molecule metabolites found in the human body. The database contains three kinds of data: (1) chemical data; (2) clinical data; and (3) molecular biology/biochemistry data. HMDB contains information on >6500 metabolites. Additionally, ~1500 protein (and DNA) sequences are linked to these metabolite entries. Because the number of metabolites that are biomarkers of metabolism is much smaller than 22,000 genes, 150,000 RNA transcripts and ~1 million unique proteins, the quantitative method of analysis of the metabolome is easier.

Metabolites are like canaries in the coal mine as indicators of human health, because the metabolome is highly sensitive to food, lifestyle, environment, seasons and even mood. A single base change in DNA can lead to a 100,000-fold change in metabolite levels. Unfortunately, <1% of known metabolites are being used in routine clinical testing. There is a need for development of tests based on metabolic biomarkers.

Glycomics

The term glycome is defined, in analogy to the genome and proteome, as a whole set of glycans produced in a single organism. Glycomics – an integrated approach to study structure-function relationships of complex carbohydrates (or glycans) – is an emerging field in in postgenomic era. Realizing the importance of glycomics, many large scale research initiatives are generate novel resources and technologies to advance glycomics, e.g. Consortium for Functional Glycomics (http://www.functionalglycomics.org/). These initiatives are generating and cataloging diverse data sets necessitating the development of bioinformatic platforms to acquire, integrate, and disseminate these data sets in a meaningful fashion.

Glycoproteomics is the study of glycoproteins, which have a predominant role in cell-cell and cell-substratum recognition events in multicellular organisms. There is increasing recognition of the importance of post-translational modifications such as glycosylation as diversifier of proteins and as potential modulator of their function in health as well as in disease.

Glycosylation is greatly affected by diseases such as cancer, and serum glycan biomarkers of various diseases have been investigated. For example, glycan biomarkers of breast cancer will be described in Chap. 13.

Single Nucleotide Polymorphisms

Small stretches of DNA that differ in only one base are called single nucleotide polymorphisms (SNP) and serve to distinguish one individual's genetic material from that of another. SNPs comprise some 80% of all known polymorphisms.

Among the roughly 3-billion nucleotide base pairs (i.e. the "letters") that make up the genetic code, SNPs occur with a frequency of one per 500 base pairs so that there are approximately 6 million SNPs. Each gene contains approximately 5 coding SNPs, which likely effect the expression of the currently estimated 30,000–40,000 genes. Identification of SNPs is important as it helps in understanding the genetic basis of common human diseases. In the absence of functional information about which polymorphisms are biologically significant, it is desirable to test the potential effect of all polymorphisms on drug response. Over nine million SNPs have been identified already. Potential uses of SNP markers are:

- Genome analysis for linkage studies
- Genome scan for association studies
- Candidate gene mapping
- Drug discovery
- Prediction of adverse effects of drugs
- Prediction of drug efficacy

Haplotyping

Haplotypes are gene versions that represent the genetic variations as they occur on each pair of chromosome in an individual – a genetic bar code with each line representing a SNP. Gene-based haplotypes are comprised of a sequence of nucleotides (approximately 25,000) that occur at SNP positions on a single chromosome at the locus of a single gene. An alternative approach to SNP genotyping is haplotyping as haplotypes contain more information than unorganized SNPs and, for practical purposes, one has to deal with a dozen or fewer haplotypes for each gene. Thus, fewer patients are needed to detect statistically significant correlation to drug response than if SNP genotyping is used alone. This forms the basic of developing personalized or individualized therapy.

Haplotypes are the most precise markers possible for a given gene because they contain all the variations in a gene. Pairs of SNPs exhibit variability in the degree of linkage disequilibrium (LD) that is a function of their location within a gene, distance from each other, population distribution, and population frequency. Haplotyping is a way of characterizing combinations of SNPs that might influence response to a drug and is considered to be a more accurate measure of phenotypic variation. However, SNP-based tests have greater power when the number of causative SNPs (a subset of the total set of SNPs) is smaller than the total number of haplotypes. One limitation of haplotyping is that haplotypes need to be determined for each individual, as SNPs detected from a pool of DNA from a number of individuals cannot yield haplotypes.

Until whole-genome sequencing of individual patients becomes feasible clinically, the identification of SNPs and haplotypes will prove instrumental in efforts to individualize health care. When an extensive inventory of genome-wide SNP scans

has been assembled across diverse population samples, LD maps using SNP and/or haplotypes will dictate that it will not be necessary to identify the precise genes involved in determining therapeutic efficacy or an adverse reaction. LD methods will provide robust statistical correlations between a patients response/risk index for a given drug class and a specific LD-SNP/haplotype profile.

Cell Biomarkers of Disease

Changes in cells may be used as biomarkers of disease. Organ-specific T cell populations can be identified by analysis of multiparameter flow cytometry data using DNA-chip analysis software. This information can be used for assessing disease progression and therapeutic intervention, and for the association of disease-related biomarkers on the protein level.

Slide-based Optical LiveCell® Array Imaging Technology (http://www.molecular-cytomics.com/) is a densely-packed hexagonal transparent array of micron-size wells that hold intact, individual cells (live or fixed) in suspension. A standard upright, inverted or confocal microscope is used with optional image analysis software. Kits are available for apoptosis and cell surface markers. Numerous applications include study of cell biomarkers and medical diagnostics.

Stem Cell Biomarkers

Scientists have been using several methods to identify the cells from different sources, including a common stem cell biomarker called CD34, but most cells identified with this method are not true stem cells. The gene, ABCG2/Bcrp1, provides scientists with a much more accurate way of identifying true stem cells as the stem cells in the bone marrow, skeletal muscle and the early mouse embryo all express the ABCG2/Bcrp1 gene in a highly specific manner. Stem cells from such diverse sources share expression of this single gene, while most mature cells showed no expression. This is particularly important because an accurate method is needed to identify stem cells the adult bone marrow; in a sample of 100,000 bone marrow cells, only one or two may be stem cells. An additional finding is that the expression of the ABCG2/Bcrp1 gene may ensure that stem cells remain in a primitive state, i.e. they do not differentiate into red blood cells, white blood cells or other kinds of cells. This finding might be useful in devising methods to control stem cell differentiation. There is also a need for identification of tissue-specific stem cells. A transgenic reporter gene approach has been used for hematopoietic stem cells (HSCs).

Association of Stem Cell Biomarkers with Disease

Identification of biomarkers associated with particular cell types can in some cases be exploited and developed into indicators of certain types of disease. The best known example is cancer stem cells. ReInnervate Ltd's research team is using mass spectrometry to identify novel biomarkers of neural stem cells. There is evidence that markers of neural stem cells are upregulated in certain forms of brain cancer. Such biomarkers may therefore also serve as potential diagnostic or prognostic indicators of neural tumors.

Cancer Stem Cell Biomarkers

The lineages assumed by stem cells during hematopoiesis can be identified by the pattern of protein markers present on the surface of cells at different stages of differentiation. Specific antibodies directed at these markers have facilitated the isolation of hematopoietic stem cells by flow cytometry. Similarly, cancer stem cells in solid organs also can be identified using cell surface markers. In addition, solid tumors contain small proportions of cells that are capable of proliferation, self-renewal, and differentiation into the various cell types seen in the bulk tumor. The tumor-initiating cells are termed cancer stem cells (CSCs) when multipotency and self-renewal have been demonstrated (Woodward and Sulman 2008). They often display characteristics of treatment resistance, particularly to ionizing radiation. CSCs may be responsible for some of the characteristics of the primary tumor in which they are found. Expression of Cripto-1 may represent a useful biomarker to identify cancer stem cells in melanoma, and possibly other aggressive tumors as well (Strizzi et al. 2008). Other examples of CSCs are given along with biomarkers of individual cancers. Biomarkers of CSCs may be useful for determining prognosis of cancer as well as targets for developing anticancer therapies.

Endoglin as a Functional Biomarker of Stem Cells

Endoglin, an auxiliary transforming growth factor receptor, plays an important role in angiogenesis and hematopoiesis, and is a functional biomarker of hematopoietic stem cells HSCs) and neural crest stem cells (NCSCs). Only NCSCs expressing high levels of endoglin have myogenic differentiation potential. Expression of endoglin in NCSCs declines with age, coinciding with a reduction in both smooth muscle differentiation potential and TGF-β1 responsiveness (Mancini et al. 2007). These findings demonstrate a cell autonomous role for endoglin in smooth muscle cell specification contributing to vascular integrity.

p75NTR as a Biomarker to Isolate Adipose Tissue-Derived Stem Cells

Adipose tissue-derived stem cells (ASCs) have been isolated and collected from mouse subcutaneous adipose tissue using the p75 neurotrophin receptor (p75NTR) as a biomarker (Yamamoto et al. 2007). Adipose tissue was processed for immunostaining using antibodies anti-CD90, anti-CD105 and anti-Sca-1 as general MSC biomarkers, and anti-p75NTR, an epithelial stem cell and MSC marker. Subsequently, the expression of cell surface markers in adipose tissue-derived stromal vascular fraction culture cells was examined by FACS. Finally, cells positive for p75NTR were sorted and cultured to induce their differentiation into adipocytes, osteoblasts, chondrocytes, smooth muscle cells and neuronal cells. Cells positive for several of these biomarkers were found in the deep layers of adipose tissue. Among them, those positive for p75NTR differentiated into adipocytes, osteoblasts, chondrocytes, smooth muscle cells and neuronal cells. The rate of differentiation into adipocytes, osteoblasts and neuronal cells was higher for p75NTR-positive cells than for p75NTR-negative cells.

Protein Expression Profile as Biomarker of Stem Cells

Differentiation of cultured stem cells often depends on the expression of genes and proteins, which could provide information on the developmental status of the cell or culture system. There are few molecules, however, that show definitive expression exclusively in a specific cell type. Moreover, reliance on a small number of molecules that are not entirely accurate biomarkers of particular tissues can lead to misinterpretation in the characterization of the direction of cell differentiation. Mass spectrometry has been used to examine proteins expressed in cultured cells as a means to identify the developmental status of stem cells and their derivatives in vitro. This approach is rapid and reproducible and it examines the expression of several different biomarkers simultaneously, providing a profile of protein expression that more accurately corresponds to a particular type of cell differentiation. Genome-wide expression profiling of ESC mRNAs and proteins and direct analyses of the cell surface subproteome have demonstrated that ESCs express a diverse range of biomarkers, cell surface antigens, and signaling molecules found in different cell lineages, as well as a number of key molecules that assure stemness of the cells (Nagano et al. 2008).

STEMPRO® EZChek™ for Analysis of Biomarkers of hESCs

The most important characteristic of human embryonic stem cells (hESCs) is their pluripotency, or ability to differentiate into all three primitive cell lineages in the body – endoderm, ectoderm and mesoderm. Scientists working with hESCs must continually screen them to ascertain pluripotency. A preferred monitoring method is

the use of PCR to amplify specific biomarkers associated with pluripotency. PCR is a sensitive and early measurement of differentiation, but it requires the sacrifice of sample cells, leaving fewer behind for research purposes.

The STEMPRO® EZChek™ Kit (ThermoFisher Scientific) enables the analysis of four early differentiation biomarkers using a small sample and a single-tube reaction. Without the kit, researchers need to carry out five separate reactions in five tubes, using many more cells. In addition, the kit requires no extensive additional training to achieve desired results.

SSEA-4 as Biomarker of MSCs

Stage-specific embryonic antigen 4 (SSEA-4), an early embryonic glycolipid antigen commonly used as a biomarker for undifferentiated pluripotent hESCs and cleavage to blastocyst stage embryos, also identifies the adult MSC (Gang et al. 2007). SSEA-4 will aid in singling out the mesenchymal stem cells (MSCs), for more detailed scientific study as well as for possible medical applications. The ability to obtain a purer, more homogeneous population of MSCs is an important consideration for applications such as tissue engineering, where only bone-generating cells are needed. SSEA-4 molecule's relationship to cancer stem cells is also being investigated. If the expression of this marker elevated in a tumor, it might be useful to identify cancer stem cells. That would be a very useful application, not only for guiding therapy, but also for early cancer detection and perhaps prevention.

Gaseous Mediators as Biomarkers of Disease

Three gaseous mediators ─ nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S) ─ are endogenously produced and act as signaling molecules in the body. They are usually associated with toxicity but several beneficial effects have also been investigated. H_2S and CO regulate a number of physiological processes, including the inflammatory response, cell death and proliferation, neural transmission and smooth muscle tone. CO, poisonous in high level exposure, can also act as a neuroprotective agent. NO is most widely studied of these molecules and is mentioned as a biomarkers in several parts of this report. Use of H_2S as a biomarker for asthma, diabetes, atherosclerosis and neurodegenerative disorders is under investigation.

Autoantibodies as Biomarkers of Autoimmune Diseases

Many human diseases are the result of autoimmune attack, presumably related to a loss of tolerance to self. Autoimmune disease can be divided into either organ-specific illnesses, such as thyroid disease, type 1 diabetes, and mysasthenia gravis, or systemic illnesses, such as rheumatoid arthritis and systemic lupus erythematosus.

Table 1.4 Autoimmune disorders under study for autoantibodies as predictors

Disease	Clinical features	Status of autoantibody research
Addison's disease	Adrenal gland insufficiency: hypotension, weakness and weight loss	Autoantibodies to adrenal tissues and enzyme 21-hydroxylase are highly predictive in children.
Antiphospholipid syndrome	Recurrent clots in blood vessels	Autoantibodies signal the risk of various complications
Celiac disease	Gastrointestinal disorder triggered by gluten in food	Predictive autoantibodies that target the enzyme tissue transglutaminase have been identified.
Diabetes type 1: insulin-dependent	Autoimmune destruction of insulin-producing pancreatic islet β cells	Autoantibodies appear years before the disease manifestations and elevated glucose in blood
Multiple sclerosis	Demyelination and multiple neurological deficits	Autoantibodies to proteins in the myelin sheaths of the nerve fibers predict the risk of disease.
Myasthenia gravis	Muscle weakness: loss of ACh receptor density at the neuromuscular junction	Autoantibodies are detected in disease but not in the pre-symptomatic phase by available tests
Rheumatoid arthritis	Chronic pain and inflammation of the joints	Autoantibodies to citrulline have been found years before the onset of symptoms
Systemic lupus erythematosus	Affecting several organs: joints kidneys and skin	Antibodies appear in 80% of the patients before onset of symptoms

The pathogenesis of autoimmune damage also segregates autoimmune disease in that some diseases or manifestations are mainly induced by autoantibodies. Pathogenesis may be mainly mediated by autoimmune T lymphocytes. Notwithstanding the underlying mechanism of disease, virtually all autoimmune diseases are associated with circulating autoantibodies, which bind self-protein. Furthermore, for many diseases these autoantibodies are found in serum samples many years before disease onset. Autoantibodies might not be directly responsible for many of the manifestations of autoimmune disease, but they are markers of future disease in presently healthy individuals. Tests that detect autoantibodies could warn of the need to take preventive action. Long-term large studies of outcome are needed to assess the use of assaying autoantibodies for prediction of disease. Autoimmune disorders that are under study for autoantibodies as predictors of disease are shown in Table 1.4. Some of these are described in more detail in chapters on various diseases.

Comparison of Various Types of Biomarkers

Comparison of various types of biomarkers is shown in Table 1.5.

Table 1.5 Comparison of various types of biomarkers

	Pharmaco-genetic	Pharmacogenomic	Toxico-genomic	Pharmaco-proteomic	Metabonomic
Parameter	Germline DNA variations	mRNA expression	mRNA expression	Protein expression	DNA, mRNA and protein interactions
Application	SNP discovery	Genotyping	Transcriptional profiling	Bioinformatics	Transcriptional profiling
Result	SNPs and complex polymorphisms	Gene expression profiles for efficacy	Pathway activation	Pathway activation	Gene expression profiles for efficacy
Prediction of efficacy	++	++++	Not applicable	+	+
Prediction of toxicity	++++	+	++++	+ +	++++

Biomarkers and Systems Biology

Systems biology is defined as the biology of dynamic interacting networks. It is also referred to as pathway, network, or integrative biology. An analysis of the structure and dynamics of network of interacting elements provides insights that are not obvious from analysis of the isolated components of the system. Types of biological networks include the following:

- Gene regulator networks
- Metabolic networks
- Protein-protein interaction networks
- Protein nucleic acid interaction networks
- Enzymes and their interactions with substrates

Systems biology requires the use of a variety of analytic platforms as well as bioinformatics, data integration, and modeling. The data can be incorporated into a mathematical framework with some predictive abilities. Ultimately, this requires a perspective on how complex systems behave and are modulated. Systems biology so far has emphasized the characterization of large pathway maps. To study the function of genes, it is necessary not only to see them in the context of gene networks, but also to reach beyond describing network topology and to embrace the global dynamics of networks that will reveal higher-order, collective behavior of the interacting genes. The combination of high-throughput methods of molecular biology with advanced mathematical and computational techniques has made it possible to screen and analyze the expression of entire genomes, simultaneously assess large numbers of proteins and their prevalence, and characterize in detail the metabolic state of a cell population. Complementing large-scale assessments, there are more subtle analyses that rationalize the design and functioning of biological modules in exquisite detail. This intricate side of systems biology aims at identifying the specific roles of processes and signals in smaller, fully regulated systems by computing what would happen if these signals were lacking or organized in a different fashion. The elucidation of this system requires high-precision, dynamic in vivo metabolite data, combined with methods of nonlinear systems analysis, and may serve as a paradigm for multidisciplinary approaches to fine-scaled systems biology.

The National Institute of General Medical Sciences (NIGMS), which has created two National Centers for Systems Biology in the US, has defined systems biology as “an integrated experimental, informational, and computational science” that has “benefited from advances in genomics, proteomics, metabolomics, and other high-throughput technologies and is driven by innovations in computational analysis and simulation.” These centers will study synthetic biology systems, multi-scale modeling approaches, signaling, genetic, and metabolic networks, and genetic variations in relation to complex phenotypes.

Systems biology is providing new challenges for advancing science and technology. Investigations based on system approach show that environmental exposures are a greater contributor to biomarker variance than are internal biological parameters

such as individual genetic makeup (Pleil 2009). The ultimate goal of this work is to develop a framework for eventually assessing the total susceptibility ranges along the toxicological pathway from exposure to effect.

Systems approaches to biomarker discovery may contribute to the discovery of more accurate and robust predictors of disease and clinical responses (Azuaje 2010). Analyses of pathways may provide new insight into the understanding of disease processes, developing more efficient biomarkers and understanding mechanisms of action of drugs. It is becoming increasingly apparent that the field of systems biology will have a major role in creating a predictive, personalized, preventive, and participatory (P4) approach to medicine (Galas and Hood 2009). It will also facilitate the transfer of technologies relevant to personalized medicine from preclinical to clinical phase.

Systems Biology Approach to Biomarker Identification

Ideally, a systematic approach to biomarker identification will involve multiple "-omic" technologies to investigate a disease process at all levels, including whole genome association studies to identify causative mutations or polymorphisms, as well as expression profiling, proteomics and metabolomics to identify expression signatures and protein and small-molecule profiles that are either specific to the disease process or provide mechanistic insights into disease pathology. Table 1.6 summarizes the use of various "omics" technologies – genomics, epigenomics/epigenetics, proteomics, glycomics and metabolomics – for discovery of biomarkers. Many of these biomarkers are interrelated in some way. Genomics is used to identify relevant disease genes, aberrant cellular signaling pathways and expression signatures correlated with disease. Proteomics is used to identify aberrant protein expression, post-translational modification, protein interactions and protein profiles that are specific to a particular disorder. Finally, metabolomics is implemented to identify the presence of abnormal levels of small molecule metabolites that are specific to and indicative of an underlying disease process.

The qualitative nature of pathology's immunohistochemical biomarkers, however, cannot be extrapolated to the realm of "omics" biomarkers, and the latter should be defined within their own paradigm preferably through a systems biology approach (Abu-Asab et al. 2011). The authors have proposed that only shared derived mutations/expressions (also known as clonal aberrations or synapomorphies) in relation to normal conditions are the potential omics biomarkers. Within the evolutionary paradigm, they demonstrated how a parsimony phylogenetic analysis models a disease onto a tree-like diagram — the cladogram that maps heterogeneous multigene expression profiles and at the same time shows the major shared clonal expressions at various levels of the hierarchical classification. Shared clonal expressions are the potential omics biomarkers that can be translated to a clinical setting in order to provide specimen characterization for early detection, diagnosis, prognosis, and posttreatment assessment.

Table 1.6 Various "omics" technologies for discovery of biomarkers

"Omics"	Sample sources	Technologies	Applications
Genomics	Nucleated cells	Positional cloning	Mapping of disease loci
	Nucleated cells	SNP genotyping	Identification of disease gene
	Nucleated cells	Microsatellites	Mapping of disease loci
	Pathologically affected cells	Expression arrays	Identification of dysregulated genes
	Pathologically affected cells	Comparative genomic hybridization arrays	Detection of gene amplification and loss of heterozygosity
Epigenetics/ epigenomics	Affected tissues	Analysis of DNA methylation Exome sequencing, bioinformatics	Biomarkers and molecular diagnostics, e.g. cancer
Proteomics	Affected tissues Body fluids: urine, blood, saliva	2D gel electrophoresis, liquid chromatography-mass spectrometry (LC-MS), ICAT-MS	Identification of protein biomarkers
Metabonomics	Body fluids: urine, blood, saliva	Nuclear magnetic resonance (NMR) MS	Identification of small molecules
Glycomics	Body fluids: urine, blood, saliva	NMR Oligosaccharide arrays	Identification of carbohydrates Identification of glycoproteins

Relation of Biomarkers to Other Technologies and Healthcare

Relation of biomarkers to other technologies and healthcare is shown in Fig. 1.1.

Biomarkers and Translational Medicine

Translational medicine deals with transfer of technologies from preclinical research into clinical application. Biomarkers play an important in translational medicine as shown in Table 1.7.

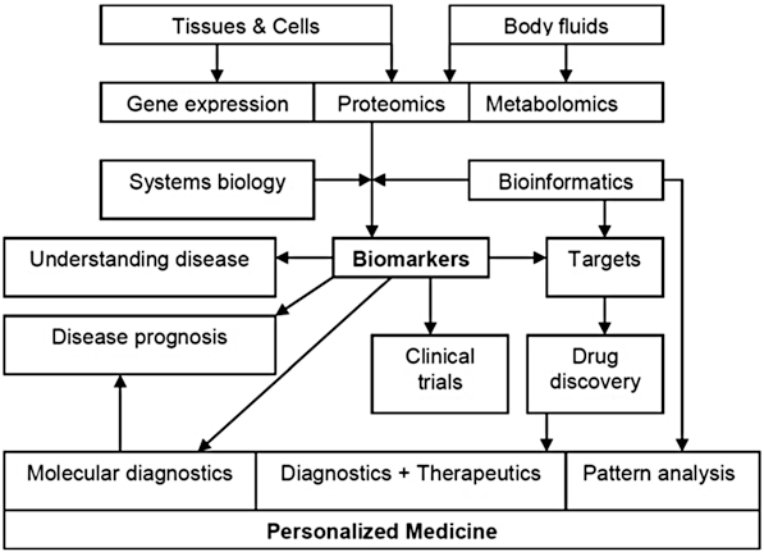

Fig. 1.1 Relation of biomarkers to other technologies and healthcare (© Jain PharmaBiotech)

Table 1.7 Role of biomarkers in translational medicine

Diagnostic biomarkers
Biomarker discovery and development into diagnostics, e.g. imaging or serum
Biomarker scoring systems to grade their predictive potency
Biomarkers for study of pathomechanism of diseases
Translational toxicology using biomarkers
Use of diagnostic biomarkers as basis for drug development
Role of biomarkers in transition from preclinical to clinical studies
Following a consistent set of biomarkers from preclinical studies to phase III trials
Using the same imaging biomarker analysis software for preclinical and clinical studies

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Role of Biomarkers in Monitoring of Diseases

Biomarker are expressed variably in the healthy population, but elevated baseline levels define increased risk for disease progression. Incorporation of biomarker analysis assists in early diagnosis, treatment choice, and early intervention, resulting in reduced disease load and time to recovery. In contrast, without biomarker monitoring, individuals at risk of disease experience both an increased disease load and longer time to recovery (Shoemaker et al. 2012).

Limitations of use of Biomarkers in Healthcare

Of the thousands of biomarkers that have been discovered and hundreds that are being reported every month, only a small number is validated and clinically useful. Several DNA biomarkers that are being reported are discovered by using and statistically flawed methods and their clinical value is negligible. Several biomarker, described as “predisposition”, “risk factor” or “susceptibility” indicators, may not be used as a basis for predictive testing because they are found too frequently in healthy people and may not develop the suspected disease although they may have a higher statistical probability that a disease will occur. Such predictive tests derived from this class of biomarker may lead physicians and patients to make medical, dietary and quality of life decisions, which may be useless. If they are used by pharmaceutical companies, the resulting drugs will not be effective in the majority of eligible patients.

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Chapter 2
Technologies for Discovery of Biomarkers

Introduction

Biomarkers are present in all parts of the body including body fluids and tissues. Most of the clinical laboratory examinations are done on body fluids such as blood and urine. Biomarkers can be detected on imaging studies or examination of body tissues. Even exhaled breath contains biomarkers. A wide range of technologies is utilized for detection of biomarkers and a number of assays are already available.

The Ideal Biomarker

From a practical point of view, the biomarker would specifically and sensitively reflect a disease state that could be used for diagnosis as well as for disease monitoring during and following therapy. Characteristics of an ideal biomarker are:

- According to the FDA, an ideal biomarker must be specifically associated with a particular disease or disease state and be able to differentiate between similar physiological conditions.
- It would be desirable if standard biological sources, such as serum and urine, could be used for identifying biomarkers.
- A rapid, simple, accurate and inexpensive detection of the relevant marker should be available, together with a measurable and standard baseline as a reference point.

An ideal biomarker should have a predictable expression level: this would demonstrate a clear association between measurable states and potential conditions.

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Genomic Technologies

A major impact of the Human Genome Project (HGP), which identified the three billion base pairs that comprise the human genome, is on molecular diagnostics. Many technologies developed during sequencing the human genome are being applied in molecular diagnostics and have led to discovery of biomarkers. Several genomic technologies used for biomarker discovery are also listed in molecular diagnostics. The presence of a gene mutation or specific gene alleles tested at the level of the patient's genome is not the same as gene expression and merely indicates that the patient is at risk for a certain disease. It does not tell us if the patient has the disease or when it may develop. Gene expression is important for this information and will be discussed here.

Gene Expression

Gene expression is used for studying gene function. Knowledge of which genes are expressed in healthy and diseased tissues would allow us to identify both the protein required for normal function and the abnormalities causing disease. Gene expression is a biomarker that will help in the development of new diagnostic tests for various illnesses. Gene expression analysis provides a means for researchers to survey and measure gene activity in various disease populations, stages of disease development, and other defined research conditions. It may also be used for safety assessment of therapeutics and chemical substances in toxicology studies, to validate drug targets, and in preclinical drug development research. Comprehensive, high information content, gene expression profiles obtained at early stages in drug discovery will ensure selection of the optimal path from target and lead to commercial product and minimize the risk of failure at later stages in development. A classification of methods of gene expression analysis is shown in Table 2.1.

Whole Genome Expression Array

The Life Technologies Corp Expression Array System is based on highly sensitive, chemiluminescent technology for gene expression detection and incorporates DNA probes that are each approximately 60 base pairs long. Chemiluminescent detection uses an enzymatic initiated chemical reaction to generate light without the need for a laser. The combination of chemiluminescence and long oligonucleotides is expected to provide higher sensitivity than other microarray products that incorporate shorter oligonucleotide lengths or use fluorescence detection methods. The long oligonucleotides enable tighter binding to the target, which leads to the detection of more genes with greater selectivity and specificity.

Table 2.1 Classification of methods of gene expression analysis

Genome-wide methods
Microarrays: Whole genome expression Array
Serial analysis of gene expression (SAGE)
Expressed sequence tags (ESTs) analysis
Gene expression profiling based on alternative RNA splicing
Tangerine expression profiling
Individual sequences
Real time RT-PCR
Competitive RT-PCR
RNase protection assay
T cell receptor expression analysis
Analysis of single-cell gene expression
RNA amplification
Monitoring in vivo gene expression
Magnetic resonance imaging (MRI)

The Life Technologies Corp's Expression Array System includes, on a single microarray, probes to detect an annotated and fully curated set of more than 20,000 human genes. This high-quality genomic information was derived from data from both public sources and Celera Genomics. Life Technologies Corp employed its sophisticated bioinformatics pipeline to design oligonucleotide probes (synthetic DNA) based on these well-defined genes for use on the microarray. In addition to the new microarray, the system comprises the 1700 Chemiluminescent Microarray Analyzer, software, and reagents. To facilitate analysis of results, the system will be combined with an industry standard database that includes gene acronyms, gene names, cross references for gene identification, gene ontologies, and protein characterization data from the Panther Protein Classification System.

The Life Technologies Corp Expression Array System will be integrated with its Sequence Detection System (SDS)-based products for gene expression analysis and the Celera Discovery System online platform. Together, these products are expected to provide an integrated solution for researchers studying changes in human gene expression levels. As a critical tool in understanding biological processes, gene expression analysis allows researchers to monitor the functional differences between normal and diseased states in order to better understand pathophysiology. This should provide researchers with a seamless solution for gene expression analysis – from whole genome analysis to further quantitation and single gene analysis.

The latest product from Life Technologies is TaqMan® Array Gene Signature Plates, which enable scientists to cost-effectively measure the activity of multiple gene targets known to be expressed in biological pathways, processes, or disease states. This allows them to discover how changes in expression levels of those genes, involved in more than 80 key biological pathways contribute to health and disease. These tools enable researchers to adopt a systems biology approach to investigate the

role gene networks play in complex biological processes and molecular pathologies by performing real-time PCR assays to develop biomarkers based on patterns of gene expression.

Gene Expression Profiling on Whole Blood Samples

Whole blood is one of the most common samples used in clinical research and the most readily available tissue for biomarker analysis. However, expression profiling on whole blood samples presents considerable technical challenges. Different whole blood preparation methods and the high relative concentration of globin RNA found in red blood cells can lead to changes in expression profiles ex vivo. Globin RNA can mask the RNA found in the transcriptionally active cells of interest in the white blood cell fraction, including the lymphocytes and monocytes. The PAXgene Blood RNA System (PreAnalytiX GmbH) has set a new standard for stabilizing whole blood cellular RNA profiles at the time of blood collection in an evacuated blood collection tube. This enables researchers and clinicians to perform more accurate analysis of gene expression profiles without the variations caused by sample collection, storage, transport or fractionation while relying on highly standardized and proven sample collection principles.

Affymetrix and PreAnalytiX have now joined forces to pursue a new standard for microarray analyses from stabilized whole blood samples to reduce variability in the preparation processes and decrease the signal contributed by globin RNA. The original information in the whole blood sample is thus preserved to enable the accurate application of molecular test methods, such as gene expression profiling.

Profiling Gene Expression Patterns of White Blood Cells

White blood cells (WBCs) express tens of thousands of genes, whose expression levels are modified by genetic and external factors. Blood genomic profiles, created from distinct gene expression patterns of WBCs obtained by microarray examination of a minimally invasive blood sample, can provide biomarkers of several different disease states. These profiles may be used for diagnostic, prognostic and therapeutic evaluations and also provide a method for the evaluation of the safety and efficacy of various therapeutics. Gene expression fingerprints are useful tools for monitoring exercise and training loads and thereby help to avoid training-associated health risks (Büttner et al. 2007).

There is marked alteration in WBC gene expression in animal models of injury and inflammation; the majority of the differentially expressed genes appear to be uniquely associated with the type of injury and/or the inflammatory stimulus. Although some pathological states such as hypoxia may have direct impact on white blood cells that is manifested by specific expression profiles, seemingly unrelated events affecting various organs can markedly alter white blood cell gene expression in a predictable, characteristic way that provides a novel approach to diagnosis of diseases such as those involving the nervous system.

Tissue Microarrays for Study of Biomarkers

Microarray experiments produce a large volume of data that require validation. The validation of these experiments can be carried out in many fashions. In the reduction to clinical utility, the use of tissue microarrays (TMAs) has become a common tool to both validate and generalize the results of microarray experiments. A TMA is a collection of tissue specimens presented on a glass slide in a grid layout. TMAs contain between tens and hundreds of samples allowing the generalization of microarray findings to a large number of samples. TMAs can be used for in situ hybridization (ISH) and immunohistochemical analysis (IHC), confirming the results of microarray experiments at both the transcriptional and the proteomic level.

The traditional IHC methods for study of biomarkers by TMAs are subjective approaches using manual analysis of tissues according to observable morphological patterns. Disparate results by these methods regarding the relationship between biomarker expression and patient outcome decrease the credibility of TMA studies. Some of these disparities result from subjective optimization of antibody concentrations. HistoRx Inc's AQUA™ (Automated Quantitative Analysis) platform dramatically improves the accuracy and speed of tissue biomarker analysis while reducing error through objective, rigorous measurement of protein expression. TMAs are discussed further in Chap. 13 (Biomarkers of Cancer).

Technologies for Detection of miRNAs as Biomarkers

Several molecular diagnostic technologies have been used for detection of miRNAs PCR, LNA, in situ hybridization (ISH) and microarrays. miRNAs are biomarkers and measurement of miRNA has diagnostic value for diseases as well, particularly cancer.

Microarrays for Analysis of miRNA Gene Expression

miRNA gene expression profiling can be done on microchips containing oligonucleotides corresponding to miRNAs from human and mouse genomes. Such microarrays have been used to obtain highly reproducible results that reveal tissue-specific miRNA expression signatures, data that is confirmed by assessment of expression by Northern blots, real-time RT-PCR, and literature search. The microchip oligolibrary can be expanded to include an increasing number of miRNAs discovered in various species and is useful for the analysis of normal and disease states. Basic analysis of miRNA genes in different mouse tissues and embryo stages using semiquantitative approaches shows tissue-restricted and developmentally restricted expression patterns of miRNAs. Newer microarray formats in development include many brain- and ESC-specific genes.

NCode™ Array (Life Technologies) enables sensitive profiling of miRNAs while using extremely simple methods that provide both experienced and novice microarray users a rapid path to new discoveries. NCode™ Rapid miRNA Labeling System (Life Technologies) combines its Alexa Fluor® fluorescent dyes with Genisphere Inc's 3DNA® dendrimer signal amplification technology, which is based on highly branched DNA structures serving as scaffolds for multitudes of fluorescent dyes. Development of this simple process to efficiently couple miRNAs directly to 3DNA® dendrimers is a significant improvement over past methods and overcomes the problem of working with small nucleic acids, where sensitivity is often limited. The new kit will accelerate the discovery of novel miRNA biomarkers. NCode™ enables researchers to study miRNA function by allowing them to profile the miRNA expression patterns in a given disease or developmental state. This will be used in molecular diagnostics and drug discovery. Sensitive microarrays to survey full sets of miRNAs in neural stem cells during differentiation, both in culture and in vivo, enabled the detection of novel patterns of regulated miRNAs systematically. An understanding of these unexpected regulatory mechanisms provides novel targets for potentially managing or controlling differentiation in stem cells prior to therapeutic transplantation.

mirVana™ System (Life Technologies) is used for miRNA microarray profiling. It involves isolation of total RNA, extraction and labeling of miRNA, and this is followed by hybridization and analysis. miRNA arrays speed up biomarker discovery and unique markers have been identified for various diseases. High-throughput detection and differential expression analysis of miRNA by microarray technology, may contribute to a greater understanding of the many events regulated by these molecules.

Microarrays vs Quantitative PCR for Measuring miRNAs

Two common methods for measuring miRNAs in a total RNA sample are microarrays and quantitative RT-PCR (qPCR). To understand the results of studies that use these two different techniques to measure miRNAs, it is important to understand how well the results of these two analysis methods correlate. Since both methods use total RNA as a starting material, it is also critical to understand how measurement of miRNAs might be affected by the particular method of total RNA preparation used.

A study involved measurement of the expression of 470 human miRNAs in nine human tissues using Agilent microarrays, and comparison of these results to qPCR profiles of 61 miRNAs in the same tissues (Ach et al. 2008). Most expressed miRNAs (53/60) correlated well between the two methods. Using spiked-in synthetic miRNAs, the two miRNAs with the lowest correlations were further examined, and the differences found could not be attributed to differential sensitivity of the two methods. Three widely-used total RNA sample prep methods using miRNA microarrays were also tested. Although almost all miRNA levels corresponded between the three methods, there were a few miRNAs whose levels consistently differed

between the different prep techniques when measured by microarray analysis. These differences were corroborated by qPCR measurements.

The correlations between results of Agilent miRNA microarray and qPCR are generally excellent, as are the correlations between different total RNA prep methods. However, there are a few miRNAs whose levels do not correlate between the microarray and qPCR measurements, or between different sample prep methods. This should be considered when comparing results obtained using different analysis or sample preparation methods.

Point-of-Care Detection of Circulating miRNAs as Biomarkers

Originally identified as intracellular modulators of protein synthesis via posttranscriptional gene silencing, miRNAs were found to travel in extracellular human fluids (circulating miRNAs) inside specialized vesicles known as exosomes. Their content inside exosomes changes during pathological events. There are >100 circulating miRNAs that can be used as biomarkers. A portable microarray POC device combining PCR and microarray platform is useful for the detection of circulating miRNAs (Vaca 2014). It reduces time for sample preparation and has enough sensitivity to detect a handful of molecules in the plasma. It is not only an auxiliary tool in diagnosis, but also useful for determining sensitivity of a patient to a particular drug in personalized medicine.

Epigenomic Technologies

Epigenomics is one of the many 'omics' that have developed in the wake of the Human Genome Project. In 2000, the Wellcome Trust Sanger Institute (Hinxton, UK) and Epigenomics AG launched of the Human Epigenome Project (HEP), a five-year undertaking during which DNA methylation sites throughout the human genome were mapped. The Human Genome Project provides the blueprint for life, but the epigenome will tell us how this whole thing gets executed, what determines when and where genes are switched on and off to produce a person. And knowing more about the human epigenome may provide clues to what goes wrong in cancer and other diseases. Latest information can be obtained at the HEP web site: http://www.epigenome.org/.

As a prelude to the full-scale HEP, a pilot study of the methylation patterns within the Major Histocompatibility Complex (MHC) has been completed. This region of chromosome 6 is associated with more diseases than any other region in the human genome. Methylation variable positions (MVPs) were identified in the vicinity of the promoter and other relevant regions of approximately 150 loci within the MHC in tissues from a range of individuals. This will provide an unprecedented insight into the complex relationship between genetics and epigenetics that underlies both

normal cellular homeostasis and disease states, in particular autoimmune diseases. For the pilot project, an integrated genomics-based technology platform was developed. The pipeline involves the automated bisulphite treatment of DNA from minute tissue biopsies, gene-specific bisulphite PCR and large-scale sequencing of PCR amplicons. Analysis and quantification of methylation patterns is achieved by mass spectrometric and microarray assays.

Discovery of Methylation Biomarkers

Methylation is the only flexible genomic parameter that can change genome function under exogenous influence. Hence it constitutes the main and so far missing link between genetics, disease and the environment that is widely thought to play a decisive role in the etiology of virtually all human diseases. Methylation occurs naturally on cytosine bases at CpG sequences and is involved in controlling the correct expression of genes. Differentially methylated cytosines give rise to distinct patterns specific for tissue type and disease state. Such MVPs are common epigenetic markers. SNPs, they promise to significantly advance our ability to understand and diagnose human disease. DNA methylation is an important cellular mechanism modulating gene expression associated with aging, inflammation and atherosclerotic processes. Global DNA hypermethylation is associated with inflammation and increased mortality in cardiovascular disease.

DNA methylation has now become one of the most studied gene regulation mechanisms in carcinogenesis. Advances in the technologies that enable detection of DNA methylation in a variety of analytes have opened the possibility of developing methylation-based tests. A number of studies have provided evidence that specific methylation changes can alter the response to different therapeutic agents in cancer and, therefore, be useful biomarkers. Application of technologies for methylation biomarkers in cancer are described in Chap. 13.

Pyro Q-CpG™ (Biotage AB) is a quantitative, high-resolution analysis solution for CpG methylation (epigenetic biomarkers), which can harmonize methylation data with observed biological phenomena. Quantitative methylSNP analysis by SNaPmeth or PyroMeth is a favorable alternative to existing high-throughput methylation assays. It combines single CpG analysis with accurate quantitation and is amenable to high throughput.

Orion Genomics' proprietary biomarker discovery platform, MethylScope® technology, is used for the discovery of methylation biomarkers. A single MethylScope® microarray is capable of quantitatively detecting the methylation status of each and every human gene. Biomarkers associated with specific diseases are discovered by comparing methylation profiles of two or more samples. MethylScope® technology is the only platform capable of detecting inappropriate DNA methylation for all human genes on a single array, providing a fast, cost-effective, and comprehensive biomarker discovery tool.

EpiTect Methyl II PCR Arrays (QIAGEN) enable the simultaneous DNA methylation profiling of a panel of 22 or 94 genes promoters using restriction enzyme based MethylScreen™ technology (licensed from Orion Genomics). Genes are carefully selected based on their reported methylation statusin a variety of experimental settings. These arrays allow correlation of CpG island methylation status with biological phenotypes or disease outcomes.

Although previous studies have described methylation of isolated DNA extracted from cells and tissues using a combination of appropriate restriction endonucleases, no application to tissue cell level has been reported. A method, called histo endonuclease-linked detection of methylation sites of DNA (HELMET), was designed to detect methylation sites of DNA with a specific sequences in a tissue section (Koji et al. 2008). In this study, the authors examined changes in the methylation level of CCGG sites during spermatogenesis in paraffin-embedded sections of mouse testis. They found hypermethylation of CCGG sites in most of the germ cells although non-methylated CCGG were colocalized in elongated spermatids. Some TUNEL-positive germ cells, which are frequent in mammalian spermatogenesis, became markedly Hpa II-reactive, indicating that the CCGG sites may be demethylated during apoptosis.

Change is DNA methylation is frequently characterized by an enzymatic modification at the 5th position of cytosine (5-mC), which is present abundantly in the context of CpG dinucleotides. The formation and maintenance of 5-mC is catalyzed by cellular DNA (cytosine-5) methyltransferases. The EpiMark 5-hmC and 5-mC Analysis Kit (New England Biolabs) offers a simple, reproducible, and robust method for examining the methylation status of a DNA population, addresses the need for detection and quantitation of 5-hmC in genomic DNAs, and opens up opportunities to determine whether 5-hmC modifications of DNA can be exploited for the identification of novel biomarkers. Furthermore, qPCR can be replaced by end-point PCR, providing scientists with a simple “yes or no” answer to whether a given locus contains 5-hmC. Finally, multiplexing of this protocol for end-point PCR may be performed for different amplicons using standard thermocyclers. EpiMark followed by qPCR is now being applied to study the differences in hydroxymethylation patterns for a variety of biologically relevant modifications in mammalian DNAs during the different stages of development. Researchers at New England Biolabs have shown variations in 5-hmC levels of genomic DNAs extracted from brain, heart and liver tissue samples from rats and humans.

Proteomic Technologies

Although protein analysis has been an integral part of clinical chemistry for decades, recent advances in technology and completion of the human genome sequencing have opened up new opportunities for analysis of proteins for clinical diagnostic purposes. New analytical methods allow the simultaneous analysis of a large number of proteins in biological fluids such as serum and plasma, offering partial views

of the complete set of proteins or proteome. Several proteomics approaches have been used to identify novel biomarkers. These are described in detail in a special report on this topic (Jain 2017b).

Not only has the number of proteins that can be detected in plasma expanded dramatically from hundreds to thousands, there is increased capability to detect structural variations of proteins. Recent studies also identified the presence of complex sets of small protein fragments in plasma. This set of protein fragments, the fragmentome or peptidome, is potentially a rich source of information about physiologic and disease processes. Advances in proteomics, therefore, offer great promise for the discovery of biomarkers that might serve as the basis for new clinical laboratory tests. There are many challenges, however, in the translation of newly discovered biomarkers into clinical laboratory tests. Only 10% of the proteins in human serum can be detected with currently available approaches, indicating the potential for further discovery of biomarkers. Protein variation is an untapped resource in the biomarker space, but only a selected few forms of proteomics applications are suitable for their analysis, and such variation could have a significant impact in disease diagnostics and therapeutic intervention.

Proteomic approaches for biomarker discovery have been used in many diseases. Two-dimensional (2D) gel electrophoresis (GE) has been used for tumor biomarker identification in cancer. Proteomics is a key technology for the discovery of biomarkers for pharmaceutical and diagnostic research. Although gene expression provides the level of proteins that is the key to the effect of the gene, it can be due to other factors in addition to the concentration of mRNA that codes for it. These factors include protein posttranslational modifications, turnover, transport and excretion. Therefore quantitative proteomics is essential for monitoring different pathways in blood samples of patients. Such biomarkers help in differential diagnosis as well as provide an understanding of pathomechanism of the disease and assessment of response to treatment. Non-invasive measurement (e.g. in serum) is the key feature of a biomarker that can be identified in diseased tissue. Multidimensional protein fractionation schemes are used to enhance sensitivity. Roche conducted a large-scale biomarker study in which the proteomic process was completely automated to generate reliable data. Using breast cancer tissue samples, several differentially expressed proteins were identified. Sandwich immunoassays were developed and serum samples from patients suffering from breast cancer were screened to discover breast cancer-specific biomarker candidates.

2D GE

2D GE offers the highest resolution separations available for protein components of cells when gels of sufficient size are used. Proteins are separated in the first dimension on the basis of their charge and in the second dimension on the basis of their molecular mass. 2D GE is still the workhorse for obtaining protein expression patterns in cells. In high-format mode, it can produce gels containing up to 10,000

distinct proteins and peptide spots. The major problem with this technique is that most of the spots cannot be sequenced as they are beyond the capacity of current high-sensitivity sequencers. Standard format 2D GE yield up to 2000 spots and are easy to sequence. During 2D PAGE (polyacrylamide GE), the proteins are separated in two dimensions (by isoelectric focusing and mass) and a pattern is achieved that places each of the 2000 proteins of the cell at a grid reference point. By reference to the databases, individual proteins on the map can be identified as the product of genes that have been sequenced.

A second generation 2D GE called Differencial In Gel Electrophoresis (DIGE) has been commercialized by GE Healthcare and involves labeling two distinct protein mixtures with two different cyanine dyes, each of which fluoresces at a distinct wavelength. The labeled protein samples are then separated on a single 2D gel. The size and charge –matched proteins enable co-migration of identical proteins.

While comparing different samples, controlling the position of the protein spots can be critical and is completely dependent upon the fidelity of the isoelectric focusing first dimension and the molecular weight separating gel slab of the second dimension. Differences between the test samples are determined by quantifying the ratios of spot intensities in independent 2D gels and techniques such as MS can then be used to help identify the proteins through peptide mass fingerprinting or direct sequencing.

ProteoCarta® Integrated Proteomics Discovery Platform

Caprion Proteomics' ProteoCarta® (formery CellCarta®) is the leading means for profiling proteins in solid tissues and plasma. This proprietary, state-of-the-art proteomics technology platform serves as an engine for both unparalleled disease target identification and predictive medicine. It is scalable and provides unparalleled sensitivity and utility. The platform has been successfully employed in oncology target identification and clinical biomarker discovery. It has four steps:

1. Sample preparation and purification. Caprion has developed novel methods for isolating organelles such as the plasma membrane, phagosomes, and golgi, to a high degree of purity. It enables more comprehensive proteomic comparisons of normal and diseased cells and provides the biological and functional context for the identification of high value, disease-relevant protein targets.
2. Mass spectrometry analysis of samples in a given project consists of two phases, an initial phase of analysis in LC-MS mode for the detection of differentially expressed peptide ions contained in the samples, and a subsequent re-injection of the same samples in LC-MS-MS mode in order to obtain sequence information for those differentially expressed peptides.
3. Quantitative expression profiling of peptides. Caprion's integrated proteomics data analysis software is capable of routinely detecting over 25,000 peptides per sample, reproducibly tracking these peptides across large sample sets and measuring their relative expression levels with high accuracy.

4. Bioinformatics analysis (protein identification).

ProteoCarta®'s core competitive advantages are:

- Its ability to comprehensively, reproducibly and quantitatively profile expression of proteins in tissues and plasma across large sample sets.
- It enables a comprehensive detection of disease-relevant protein targets and of biomarkers across a wider dynamic range of concentrations.
- It produces a complete audit trail and data analysis/validation toolset.

Applications relevant to discovery of biomarkers are as follows:

- Pharmacodynamic biomarkers for drug efficacy, dose determination, mechanism of action, and safety.
- Disease biomarkers for diagnostics and surrogate for clinical response to therapy
- Predictive biomarkers for patient stratification, drug efficacy and safety

Isotope-Coded Affinity Tags

Isotope-coded affinity tag (ICAT) peptide labeling is an approach that combines accurate quantification and concurrent sequence identification of individual proteins in complex mixtures. This method is based on a newly synthesized class of chemical reagents used in combination with tandem mass spectrometry. The method consists of four steps:

- The reduced protein mixtures representing two-cell state are treated with two different versions of ICAT reagent – one light and one heavy.
- The labeled sampled are combined and proteolytically digested to produce peptide fragments.
- The tagged cysteine-containing fragments are isolated by avidin affinity chromatography.
- The isolated tagged peptides are separated and analyzed by microcapillary tandem MS which provides both identification of peptides by fragmentation in MS-mode and relative quantitation of labeled pairs by comparing signal intensities in MS mode.

The advantages of ICAT over 2D GE that has the potential for full automation, and thus for high-throughput proteomic experiments. There is no need to run time-consuming experiments and because it is based on stable isotope labeling of the protein, there is no need for metabolic labeling or no radioactivity is involved. ICAT can be used for the analysis of several classes of proteins such as membrane proteins and low copy number proteins that are poorly tractable by 2D gels. Most importantly, it provides accurate relative quantification of each peptide identified. The limitations of this technique are that the proteins must contain cysteine and the large size of the tag compared to some small peptides and may interfere with peptide

ionization. These, however, can be overcome by designing different reagents with specificities for other peptide chains and using a smaller tag group.

ICAT is a technique for differential expression proteomics, and its full potential remains to be fully evaluated. Advances in sample fractionation at the protein level, sample fractionation at the peptide level, and improved data acquisition schemes, will all be required for the full potential of ICAT to be realized. New separation systems, such as ultra-high pressure nanoscale capillary LC will improve the peak capacity for ICAT experiments, leading to improved proteome coverage. New MS technologies, such as the high sensitivity, high-throughput MALDI-TOF instrument, can be expected to have a very significant impact in ICAT proteomics.

Liquid Chromatography-MS/MS

Classical LC (liquid chromatography) has been combined with MS. However, some emerging strategies are shown to be more suitable for protein characterization and identification. Microanalytical protein characterization with Ettan multidimensional liquid chromatography (GE Healthcare) achieves reproducible separation of proteins based on more than one physical property. MDLC /mass spectrometry (MDLC/MS) improves the throughput and reliability of peptide mapping for the following reasons:

- Faster protein digestion with immobilized enzymes
- Automated generation of peptide maps
- Online detection of peptide maps by electrospray interface and MALDI-TOF (matrix-assisted laser desorption ionization mass spectrometry).

Lucid Proteomics System

The Lucid Proteomics System (Bio-Rad) combines and refines surface-enhanced laser desorption/ionization (SELDI) and time-of-flight (TOF/TOF) technologies from Bruker, enabling both top-down and bottom-up proteomics approaches for biomarker discovery in one system (Jourdain et al. 2010). The system offers new solutions for protein biomarker discovery, in particular, high-throughput profiling and high-confidence identification of intact peptides and proteins under 30 kilodalton, which are challenging for current technologies to identify.

Magnetics Beads for Protein Biomarker Discovery

Magnetic beads are providing ways to identify protein biomarkers faster, more efficiently and at lower cost than other methods. ProMag™ (Bangs Laboratories) is a magnetic polymer 3μ sphere, which has a hydrophilic surface to reduce nonspecific

binding in protein-based systems. The 3μ size provides an ample surface for capturing/purifying targets. For biomarker discovery, investigators can coat beads with ligand of interest.

MASStermind™

MASStermind™ (MyCartis), a biomarker discovery engine, is based on novel proteomics technologies developed at the Department of Medical Protein Research at the University of Ghent, Belgium and the Flanders interuniversity Institute of Biotechnology. These proprietary proteomics technologies allow fast, automated and highly flexible and sensitive qualitative and quantitative proteomics without using gels and without the use of an affinity tag. The key difference from currently available profiling technology is MASStermind's high information content; it delivers both a profile and the identity of each of the underlying protein biomarkers. Analysis of one blood sample allows the quantitative assessment of ~3000 different proteins and their processed isoforms in a high throughput fashion. The basic strategy comprises the following steps:

- Isolation of proteins from a biological sample
- Cleavage of proteins to peptides
- Chromatographic fractionation of the complex peptide mixture
- Modification of a target subset of peptides
- Specific isolation of the modified peptides by a second automated chromatography
- Analysis by mass spectrometry of the isolated peptides and
- Protein identification via intelligent interrogation of databases with novel software.

The procedure, in which fractions of the first chromatographic step are combined, modified and run in a diagonal chromatographic manner, is called COmbined FRActional DIagonal Chromatography (COFRADIC™).

Combined Analysis of Protein and Nucleic-Acid Biomarkers

Evalution™ is MyCartis' multiplex analysis platform tailored to the development of clinical biomarkers. It analyzes a broad range of biomarkers to delivers high quality data and rapid results from minimal handling steps with unprecedented ease. Evalution™ integrates into a single instrument all the functions of incubation, washing, and optical readout for seamless operation of sophisticated assay protocols. The combination of customizable encoded microparticles with microfluidic assay plates in Evalution™ enables the combined analysis of protein and nucleic-acid biomarkers.

Mass Spectrometry

Mass spectrometry (MS) is the measurement of molecular mass. A mass spectrometer consists of three essential parts: (1) an ionization source with conversion of molecules into gas-phase ions; (2) a mass analyzer to separate individual mass to charge rations (m/z); and (3) an ion detector. Several variants of mass spectrometry are described in the following sections. Proteomics methods based on MS hold special promise for the discovery of novel biomarkers that might form the foundation for new clinical blood tests, but to date their contribution to the diagnostics has been disappointing, partly due to the lack of a coherent pipeline connecting marker discovery with well-established methods for validation. Advances in methods and technology now enable construction of a comprehensive biomarker pipeline from components: candidate discovery, qualification, verification, research assay optimization, biomarker validation and commercialization.

Biomarker discovery using MS techniques requires sensitivity, mass accuracy and reproducibility. Various MS-based techniques used for biomarker discovery are described in the following text. The central role of mass spectrometry in proteomics is shown in Fig. 2.1.

2D PAGE and Mass Spectrometry

The essence of this approach is to separate proteins from a specific cell or tissue type, record the pattern, and then produce a Western Blot. Proteins in the blot are digested with a proteolytic enzyme, which has well-defined cleavage specificity.

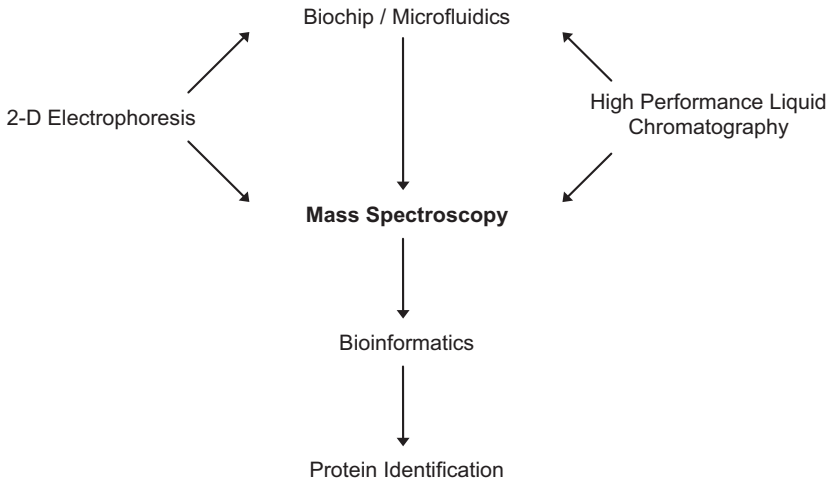

Fig. 2.1 The central role of spectrometry in proteomics (© Jain PharmaBiotech. Reproduced from the same number figure in the 1st edition)

Peptide fragments can be analyzed by Matrix-Assisted Laser Desorption Mass Spectrometry (MALDI-MS). The resulting peptide masses are then compared with theoretical masses calculated from amino-acid sequence databases. This technique has been used successfully to identify yeast proteins. For completely sequenced genomes, 90% of the proteins can be identified rapidly and automatically by searching databases with lists of peptide masses obtained by 2-D gel technique and matrix-assisted laser description ionization. This study established that mass spectrometry provides the required throughput, the certainty of identification, and the general applicability to serve as the method of choice to connect genome and proteome. Nanoelectrospray tandem mass spectrometry is then used to characterize novel proteins either by searching EST databases with peptide sequence tags, or by generating sufficient sequence for cloning. This approach can be automated.

Imaging Mass Spectrometry

Imaging MS (IMS) is an emerging technology that allows the direct analysis and determination of the distribution of molecules in tissue sections. Pinpointing the location of the cells producing different levels of specific proteins enables the identification of differences in normal and diseased tissues that could lead to a better understanding of the onset and treatment of disease. This approach by combining MALDI MS for protein identification with visual tissue and cellular imaging can be used to determine the location of specific proteins in different tissue sections of diseased and normal organs.

IMS is a combination of MS with tissue imaging. Molecular imaging is achieved through secondary ion mass spectrometry (SIMS), which has unique analytical capabilities for mapping a variety of biological samples at the tissue level. SIMS provides information on the spatial distribution of the elements and low molecular mass compounds as well as molecular structures on these compounds, while MALDI yields spatial information about higher molecular mass compounds, including their distributions in tissues at very low levels, as well as the molecular structures of these compounds. To take molecular photographs, a chunk of tissue is first frozen so that it can be cut into super-thin slices. A tissue slice to be analyzed is then coated with a matrix material and introduced into the mass spectrometer, where a laser beam blasts successive spots on the tissue to release molecules for analysis. Each spot becomes a pixel in the final image, with each pixel containing a record of the molecules located in that tiny spot. Computer processing can then be used to display the locations of selected proteins, based on their size.

Separation of shotgun-produced peptides by the use of Immobilized pH Gradient-IsoElectric Focusing (IPG-IEF) has been combined with IMS (Vaezzadeh et al. 2008). The peptides are then transferred by capillarity to a capture membrane, which is then scanned by the mass spectrometer to generate MS images. This high-throughput method enables a preview of the sample to be obtained in a single day and has been used for differential comparison of the membrane proteome of two different strains of *Staphylococcus aureus* bacteria in a proof-of-principle experiment.

Biological molecules such as proteins, peptides, lipids, xenobiotics, and metabolites can be analyzed in a high-throughput manner with molecular specificity not readily achievable through other means. Tissues are analyzed intact and thus spatial localization of molecules within a tissue is preserved. IMS has the potential to deliver highly parallel, multiplexed data on the specific localization of molecular ions in tissue samples directly, and to measure and map the variations of these ions during development and disease progression or treatment. It is possible to identify the biomarkers in the same experiment, or by relatively simple extension of the technique. Unlike many other imaging techniques, no a priori knowledge of the biomarkers being sought is necessary.

A publication presents several studies that focus on the unique types of information obtainable by IMS, such as Aβ isoform distributions in Alzheimer's plaques, protein maps in mouse brain, and spatial protein distributions in human breast carcinoma (Seeley and Caprioli 2008). The analysis of a biopsy taken 100 years ago from a patient with amyloidosis illustrates the use of IMS with formalin-fixed tissues. The technique can also assist in tracking the location of drugs and their metabolites in the treatment of various disorders, including cancer, thus providing a potentially powerful new research tool.

MALDI Mass Spectrometry for Biomarker Discovery

Matrix-Assisted Laser Desorption/Ionization MS (MALDI-MS) has become a widely used method for determination of biomolecules including peptides, proteins, carbohydrates, glycolipids and oligonucleotides. MALDI-MS has emerged as an effective bioanalytical tool having unique capabilities in handling complex mixtures (such as proteolytic digests) and in high-sensitivity (femtomole or even subfemtomole) measurements. Direct analysis of tissue sections by MALDI-MS has a tremendous potential for biomarker discovery, and routinely enables hundreds of proteins to be detected over a mass range of ~2000 to 70,000 Da while maintaining the spatial localization of the proteins detected. MALDI-MS has been applied to a wide range of tissue samples, including human glioma tissue and human lung tumor tissue. In many cases, biostatistical analyses of the resulting protein profiles revealed patterns that correlated with disease state and/or clinical endpoints.

MS platforms using MALDI MS and MS/MS or LTQ ion trap MS are capable of delivering sensitive and accurate identifications of hundreds of proteins contained in individual samples including individual forms of processing intermediates such as phosphopeptides. The Systems Biology approach of integrating protein expression data with clinical data such as histopathology, clinical functional measurements, medical imaging scores, patient demographics, and clinical outcome provides a powerful tool for linking biomarker expression with biological processes that can be segmented and linked to disease presentation.

MALDI-TOF MS is a particle-counting method that responds to molar abundance, and ranking of plasma proteins by molar abundance increases the rank of small proteins relative to traditional ranking by mass abundance. Detectors for

MALDI-TOF MS augment the bias for detecting smaller components by yielding stronger signals for an equivalent number of small vs large ions. Consequently, MALDI-TOF MS is a powerful tool for surveying small proteins and peptides comprising the peptidome or fragmentome, opening this new realm for analysis. It is complementary to techniques such as electrophoresis and HPLC, which have a bias for detecting larger molecules. Virtually all of the potential markers identified by MALDI-TOF MS to date represent forms of the most abundant plasma proteins. Analyses of serum or plasma by MALDI-TOF MS thus provide new information mainly about small proteins and peptides with high molar abundance. The spectrum of observed proteins and peptides suggests value for applications such as assessment of cardiovascular risk, nutritional status, liver injury, kidney failure, and systemic immune responses rather than early detection of cancer. Extending analysis by MALDI-TOF MS to lower abundance components, such as biomarkers for early-stage cancers, probably will require more extensive specimen fractionation before analysis.

Quantitative Tandem MS

Mass spectrometers are making large inroads into the modern day life science industry. Their high-throughput capability, resolution and precision have ensured their place in pharmaceutical industry and life science research institutes as pivotal tools to screen hundreds and thousands of DNA and protein samples per day. The advantages are obvious when proteins or nucleotides can be rapidly analyzed in a few seconds by MALDI.

Modern tandem mass spectrometers have many benefits. Amongst these is the ability to accurately measure down to femtomolar amounts in complex biological mixtures without chromatography. This enables samples to be prepared and analyzed using high-throughput technologies such as robotic liquid handlers and microtiter solid phase extraction systems. The operator of tandem mass spectrometry (MS/MS) experiments can choose metabolite specific fragmentations for definitive analytic identification. Thereby, arrays of metabolites in multiple pathways can be accurately identified and quantified per sample run akin to DNA and protein multiplexing technologies.

Single-Molecule Mass Spectrometry Using a Nanopore

Interaction between a nanometer-scale pore and analytes can be used for mass spectrometry (MS) in solution. For example, poly(ethylene glycol) molecules that enter a single α-hemolysin pore cause distinct mass-dependent conductance states with characteristic mean residence times. The conductance-based mass spectrum clearly resolves the repeat unit of ethylene glycol, and the mean residence time increases monotonically with the poly(ethylene glycol) mass. This single-molecule analysis technique could prove useful for the real-time characterization of biomarkers

(i.e., nucleic acids, proteins, or other biopolymers). With automated, unsupervised analytical and statistical methods, this technique should prove viable as a generalized analytical technique with nanopore arrays containing nanopores both with specific affinities for single biomarkers and with nonspecific transducers. In situ monitoring of cellular metabolism with such arrays should provide the sensitivity to monitor subtle changes observed through the release of biomarkers.

Requirements for MS-Based Proteomic Biomarker Development

The success of the MS process is critically dependent on the ability to identify as many putative active or informative molecules as possible and having efficient methods to weed out weak candidates in subsequent steps. In biomarker development this requires definition of performance goals, the ability to test these goals in relevant populations, and the ability to evaluate the performance of multianalyte panels and discover covariance in addition to single markers. Some requirements for successful MS-based proteomic biomarker development are:

Samples. These should be collected, preprocessed and stored in a uniform way, but should be split into two sets. The validation sample sets are identical to training samples but used only for validation.

Separation. Discriminatory peaks must be identified to assess relevance for intended use and to develop immunoassays.

Statistics. Tools used include support vector machines, discriminant analysis, classification trees, random forest, and neural networks.

Nucleic Acid Programmable Protein Array

Nucleic Acid Programmable Protein Array (NAPPA) technology, originally developed at the Harvard Proteomics Institute, enables the expression of up to 7500 different human proteins at high concentrations and in a biologically active form. Studies indicate that NAPPA provides up to 1000 times more protein per spot than levels attainable with conventional protein microarrays. Moreover, NAPPA proteins are synthesized in situ on the array surface, avoiding the need for individually expressing, purifying and printing thousands of individual proteins.

NAPPA has a wide variety of applications including the study of protein-protein interactions, protein-drug interactions and the identification of biomarkers. Proteomika has been applying NAPPA technology to the identification of auto-antibodies present in cancer patients as potential disease biomarkers. By identifying common cancer antigens that trigger antibody production, it is possible to establish characteristic autoimmune profiles allowing the identification of auto-antibodies that can be used as early biomarkers for certain types of cancer.

Protein Tomography

Light microscopy techniques can also provide images of cellular events. However, these techniques do not show structure and function on a protein level. NMR, X-ray and single particle electron microscopy provide higher resolution structural information but the proteins are studied in an artificial environment. In addition, the protein image is compiled of an average of thousands to millions of objects. As a result, the latter methods are unable to give information about flexibility and shape of individual proteins or protein complexes as they are not in situ.

Sidec Technologies has developed Protein Tomography™ (formerly called Sidec Electron Tomography), which is basically the molecular biology of medical imaging. It can be used for imaging of molecular events in situ or in vitro, combining cryo-EM, electron tomography and advanced data processing algorithms. It can accomplish the following:

- It can take images of individual proteins and protein complexes.
- It is the only method that picture membrane proteins in their cellular context.
- The results are directly comparable between in vitro and in situ analysis.
- It enables visualization of the different states of the same molecule in a mixture: unbound receptors, receptors attached to ligands, recruitment of signaling proteins, etc.
- It is an ideal method for study of proteins as biomarkers.

Protein Biochips/Microarrays and Biomarkers

Protein microarray technology is a powerful tool for biomarker discovery and validation. Protein microarrays have this potential and it will, in future, be possible to use them to determine simultaneously a variety of parameters from a minute sample volume, as well as in the discovery and validation of biomarkers. However, before protein microarrays find their way into routine and high-throughput applications, their robustness, sensitivity and automation must be tested thoroughly and they must be available at an affordable price. Nevertheless, for focused protein-profiling approaches searching for only a few parameters in parallel, current technologies are already mature enough to deliver reliable datasets.

ProteinChip (Vermillion) has been used for discovery of biomarkers. The advantage of ProteinChip over 2D GE is that the chip platform used to identify the biomarker can also be used to develop a high-throughput assay. Laser capture microdissection has been used in conjunction with ProteinChip to study protein expression profiles in cancer.

Antibody Array/Affinity Proteomics-Based Biomarker Discovery

Affinity proteomics, based on the use of antibodies, has emerged as a tool capable of gathering information on the global level in a high-throughput format. An affinity proteomics approach is being used to explore the human proteome under the Swedish Human Proteome Atlas project (http://www.proteinatlas.org/). A 2-step affinity chromatography principle was devised to first deplete the affinity tag-specific antibodies followed by a second step for affinity capture of the target protein-specific antibodies. An analytical dot-blot array system was developed to analyze the cross-reactivity of the affinity-purified antibodies. The results suggest that the protocol can be used in a high-throughput method known as protein epitope signature tags (PrESTs). Tissue arrays (comprising healthy and diseased tissue and a large number of different cell lines) containing characterized monospecific antibodies were used to screen for the expression of corresponding proteins. The specificity of the staining could be determined using two antibodies that bind to different epitopes of the same protein. Antibodies derived by Human Protein Atlas can also be used for high-throughput biomarker analysis via IHC on tissue microarrays.

Multiple versions of affinity reagents are deployed in affinity proteomics, including full-length antibodies, aptamers, affibody molecules, and single-chain variable fragments of antibodies. Various capture formats are also being explored, ranging from planar arrays (similar to commonly used printed DNA arrays) to beads, to direct synthesis of antibodies in the array format. Affinity arrays are rapidly emerging as powerful biomarker discovery tools. Antibody-based proteomics has already delivered multiple specific biomarker candidates, and many of those are being translated into clinical practice.

Antibody arrays (also called protein expression microarrays) can be used for screening of biomarkers. As in the case of gene expression microarrays, it is expected that protein expression microarrays will help in biomarker discovery, prediction of disease outcomes and response to treatments, and detection of molecular mechanisms and/or pathways associated with a particular disease state. However, accurately achieving these aims is dependent upon suitable experimental designs, normalization procedures that eliminate systematic bias, and appropriate statistical analyses to assess differential expression or expose expression patterns. A considerable amount of research has been devoted to two-color cDNA arrays to improve experimental design, normalization and statistical analyses to assess differential expression and classification. These methods are directly applicable to two-color antibody arrays. Statistical methods that have been developed for cDNA arrays have been applied to antibody arrays as well.

RayBiotech Quantibody®7000 sandwich-based array offers global profiling of 320 unique proteins related to cytokine signaling. RayBiotech antibody arrays are used for probing disease processes, particularly when only a few details are known, e.g. use of a broad screening array confirmed the long-suspected role of inflammation in autism by detecting high expression of inflammatory cytokines in brains of

autistic patients. RayBiotech's C-1000 120-cytokine screening array was used to profile plasma from patients with the confirmed Alzheimer disease (AD) diagnosis and from matched controls. Prediction analysis and cross-validation established an 18-biomarker signature, which was able to correctly predict clinical diagnosis of AD with 89% accuracy.

Using antibody capture with single-molecule detection technique, as few as 600 molecules per spot can be detected and counted. This method has been used to develop a proof-of-concept diagnosis of tuberculosis based on capture and detection of lipoarabinomannan, a polysaccharide biomarker of tuberculosis. Current tuberculosis diagnosis is not sensitive enough to detect the infection at early stages when the treatment may be the most effective. With the rapid re-emergence of tuberculosis worldwide, a reliable and sensitive diagnosis is of critical importance for disease control. Antibody capture combined with fluorescence detection seems to be well positioned to address this growing need.

The resolution of proteomic analyses by antibody arrays is, however, directly related to the number of data points, i.e. antibodies, included on the array. Currently, this is a key bottleneck because of limited availability of numerous highly characterized antibodies. A conceptually new method has been presented that denotes global proteome survey, and opens up the possibility to probe any proteome in a species-independent manner while still using a limited set of antibodies (Olsson et al. 2011). Context-independent-motif-specific antibodies directed against short amino acid motifs are used, where each motif is present in up to a few hundred different proteins. First, the digested proteome is exposed to these antibodies, whereby motif-containing peptides are enriched, which are then identified by MS. The authors of this study have profiled extracts from human colon tissue, yeast cells lysate, and mouse liver tissue to demonstrate proof-of-concept.

PlasmaScan™ (BioSystems International) and QuantiPlasma™ MAb libraries (Randox Laboratories) cover the entire human plasma epitome and provide the tools that enable the discovery of new disease specific biomarkers via robust antibody microarray profiling of human plasma samples. PlasmaScan 80/PlasmaScan380 antibody arrays contain 80 and 380 (respectively) different MABs each reacting with different epitopes of the human plasma proteome. MAbs recognize native protein structures and post-translational modifications (Wang et al. 2010). The characterization of MAbs in this work reveals that the global MAb proteomics process can generate high-quality lung cancer specific MAbs capable of recognizing proteins in their native state. QuantiPlasma™ antibody library enables precise proteome profiling without sample labeling. The antibodies work in "tracer inhibition assays" using stabile labeled tracers for the profiling experiments, which are included in the kits.

Detection of Biomarkers Using Peptide Array Technology

Peptide arrays can be used to diagnose almost every disease providing their biomarkers are available. Peptides recognizing those biomarkers can be synthesized on a single array and one test can simultaneously screen for all biomarkers.

High throughput and high resolution make it possible to distinguish the slightest difference between two disease biomarkers. In addition, unlike DNA arrays, the detection of disease biomarkers on a peptide array is technically very straightforward and easy to operate. Therefore, the peptide array can provide a powerful tool for clinical diagnosis in the future.

ProtoArray®

ProtoArray® Human Protein Microarray (Life Technologies) is the first high-density microarray and contains thousands of unique, full length human proteins including kinases, phophatases, GPCRs, nuclear receptors, and proteases, spotted in duplicate on a 1 × 3 inch thin nitrocellulose coated glass slide. ProtoArray® human protein microarray version 5.0 contains over 9000 unique human proteins individually purified and arrayed under native conditions to maximize functionality. Version 5.0 also includes control ProtoArray® Human Protein Microarray proteins in each sub-array to facilitate supported applications. Additionally, data analysis software, ProtoArray® Prospector, is freely available for examining the results. The advantages of this technique are:

- Results in as little: as one day
- Identification of biomarkers with as little as 10 uL of serum
- Simple protocols to profile over 9000 proteins with a variety of samples

Applications of ProtoArray® Human Protein Microarray are:

- Immune response biomarker profiling
- Small molecule profiling
- Enzyme substrate profiling
- Antibody specificity profiling
- Protein-protein interaction profiling

Protein Nanobiochip

Biomarker proteins as early warning signs for diseases such as cancer can be identified for diagnostic purposes by finding their isoelectric points and their molecular weights. Isoelectric points are chemical features that refer to the electrical state of a molecule when it has no net charge. Conventional protein chips use a gel across which an electric current is applied to find the targeted protein's isoelectric points. In the nanobiochip, instead of being filtered through a block of gel, the protein molecules are separated by their isoelectric points by a capillary action as the proteins flow in a solution along channels in the chip. The protein molecules are then dried and irradiated by a laser. Their molecular weights are then measured by a mass spectrometer. The laser helps the proteins leave the chip, and the mass spectrometer is used to judge the molecular weights of the protein molecules in the samples by

measuring how early they reach a detector. In the mass spectrometer, light molecules fly faster than heavy ones in an electric field. The mass spectrometer judges the weight of the molecules by monitoring the timing of when each molecule reaches a detector. In addition to being faster than techniques that use gel blocks, the nanobiochip method requires blood samples of about 1 μL compared to about 20 μL or more that are needed using gel-based techniques.

Gene Expression Microarray Data as a Source of Protein Biomarkers

Gene expression microarrays have been in use for more than a decade ago, and microarray studies have been used to study changes in mRNA transcripts in disease-related tissues. Considerable microarray data have been deposited into international repositories including the Gene Expression Omnibus (GEO) and ArrayExpress. Integration of publicly-available microarray data has been used to detect candidate gene variants common to various types of cancers and even for to validating gene-expression-based diagnostics for FDA approval. Although microarrays measure the relative abundance of mRNA transcripts, their translated proteins are also likely to be differentially present in diseased tissue and possibly even secreted or detectable in the blood. A method was described to determine gene expression signatures across 238 diseases from GEO and revealed that the molecular signature of disease-specific RNA across tissues is more prominent than the signature of tissue-specific expression patterns (Dudley et al. 2009). Thus RNA measurements in diseased tissue could be used to identify candidate serum protein biomarkers of disease.

Quantification of Protein Biomarkers

Multiple Reaction Monitoring Assays

Multiple reaction monitoring mass spectrometry (MRM-MS) assays, originally developed to quantify small molecules, is now being adapted for quantification of protein biomarkers. Analytical characteristics of the MRM-MS approach make it ideally suited for the clinical laboratory as an assay for biomarker tests. MRM-MS techniques have been used clinically for quantification of protein biomarkers, including biomarker panels (Hunter and Paramithiotis 2010). Among the different methods to read out quantitative signals by MS to measure specific proteins of interest, MRM has proven to be most suitable, with a wide linear range (Schmidt and Urlaub 2012). Because of multiple measurements per protein, MRM is one of the most specific assays for protein measurement. However, targeted analysis by MRM requires the individual design of suitable assays. A number of companies have developed commercial MRM-based assays.

Caprion Proteomics' MRM assays use triple quadrupole MS coupled to LC. Protein biomarkers can be selected from genomics, literature, or discovery efforts. In the first quadrupole, a specific peptide that corresponds to a protein of interest is selected. The peptide is then fragmented in the second quadrupole and a filter is applied to allow a specific fragment, also referred to as a transition, to enter into the third quadrupole where its intensity is measured. Typically, two peptides per protein are monitored and two transitions per peptide are measured, which improves signal-to-noise and reduces interference. In addition, current instrumentation enables measurement of hundreds of proteins in a single sample, making MRM an ideal assay to verify and validate candidate biomarkers or perform high-throughput measurements of a panel of biomarkers.

MRM Proteomics' PeptiQuant™-based MRM assay panels and analytical services are optimized to enable the most sensitive, accurate, and specific results while providing the ability to measure the exact concentration of each protein in complex matrices such as blood/plasma directly. It enables targeted analysis of proteins of interest rather than sifting through enormous amounts of data that typically result from non-targeted studies.

Indi (Integrated Diagnostics Inc) Molecular, a emerging life sciences company, is developing a synthetic class of diagnostic and therapeutic agents with antibody-like properties, protein-catalyzed capture agents (PCCs), which were created in collaboration with the California Institute of Technology that enables scientists to permanently join together molecular components with unusual precision and stability. PCCs offer superior stability, lower cost and faster creation compared to MAbs for identifying biomarkers for diagnostics and therapeutics.

Real-Time PCR for Quantification of Protein Biomarkers

The TaqMan Protein Expression Assays (ThermoFisher Scientific) are a new line of TaqMan® real-time PCR assays that enable researchers to rapidly detect and quantify proteins in human cell samples as well as correlate relative levels of specific proteins with cell functions in different disease conditions. The new assays detect and quantify proteins by an innovative technology that combines an antibody-oligonucleotide-tagged immunoassay with a TaqMan Assay to generate real-time PCR data for specific proteins present in as little as 10–250 cells. These tools offer researchers a more quantitative, simpler, and standardized approach to protein analysis of various cell types, especially stem cells, compared to other more complex methods that also require large amounts of cell sample. When combined with TaqMan Assays for miRNA and mRNA and run on one of Life Technologies' real-time PCR systems, they form the only quantitative protein analysis method that enables researchers to make comparisons of protein and RNA molecular biomarkers identified on the same platform with the same starting samples.

The new assays for targets in stem cells have already been used by a team of scientists at Erasmus University Medical Center (Rotterdam, The Netherlands) to quantify protein biomarkers for pluripotency (OCT 3/4, NANOG, SOX2, and

LIN28) using small quantities of human stem cell and testicular germ cell samples. These types of biomarkers can potentially be used to identify and characterize malignant cells.

CyTOF for Quantification of Biomarkers

CyTOF (DVS Sciences Inc/Fluidigm) is a mass cytometer combining flow cytometry and atomic mass spectrometry that can quantify biomarkers in parallel in numbers not achievable with other technologies. Initially developed as an improvement on current flow cytometry instruments, the technology has been adapted for a multiplexed bead array instrument. Instead of using fluorescence tags or lasers, it uses a stable isotope of an element, preferably a stable lanthanide isotope, which is attached to an antibody. The cells are probed and then flowed one at a time into the mass spectrometer, which vaporizes, atomizes, and ionizes the cell. The ions are extracted from the resulting state-of-the-matter plasma and analyzed by mass spectrometry at about 1000 cells per second. The results are quantitative, because the atomic mass spectrometer does complete ionization. The signal recorded at the detector is exactly proportional to the number of atoms that go in the front end, the number of antibodies that were present, and the number of antigens in the cell. The instrument provides the ability to measure several antigens simultaneously and quantitatively.

CyTOF takes flow cytometry into the next dimension of the multiparameter stage and it does it without requiring a lot of calculations and corrections, Maxpar reagents, developed for use on CyTOF, can also be used with conventional ICP-MS analysis for solution analysis, such as multi-parametric ELISAs or Western blot format. The advantage of the CyTOF over conventional flow cytometers is that the mass spectrometer enables exquisite resolution between the mass channels without overlap, Therefore, one can do as many measurements as there are stable isotopes and for which there is a non-cross-reactive antibody panel. CyTOF allows one to do a 35-parameter experiment because there are 35 lanthanide stable isotopes that they use as tags, making it the first instrument that allows flow cytometry to be done in >20 parameters. Currently, CyTOF is for use with protein and RNA biomarkers and is suited for targeted biomarker work. The technology is amenable to any affinity assay as long as there is an affinity product that recognizes the target – a hybridization gene array, an antibody assay, an aptamer, a lectin or a hapten. It has an impact on drug development lifecycle in deciphering a drug's pharmacokinetics and pharmacodynamics relationship (Atkuri et al. 2015).

Search for Biomarkers in Body Fluids

The first decision to be made in a search for a biomarker is whether to look in a body fluid or a tissue. Body fluids have the advantage of being more easily accessible and are more likely to be of clinical use because serum or urine can be obtained by

non-invasive methods as a routine (Jain 2007). However, plasma (together with serum) is the largest and deepest version of the human proteome and presents challenges for the investigators.

Challenges and Strategies for Discovery of Protein Biomarkers in Plasma

Plasma is the most difficult sample to work with in proteomics, despite the relatively good behavior (i.e. solubility) of its protein components. The daunting size of the plasma proteome is a reflection of the sheer number of different proteins to be tested – $>10^6$ different molecules representing products of all 25,000–30,000 genes. Approximately half of the total protein mass in plasma is accounted for by only one protein (albumin, present at ~55,000,000,000 pg ml^{-1}), while roughly 22 proteins, which also include transferrin, fibrinogen and other, make up 99% of the total. The remaining 1% contains secreted proteins, some of which are promising biomarkers. At the other end of the concentration histogram are the cytokines, such as interleukin-6 (IL-6), which is normally present at 1–5 $pgml^{-1}$. The difference in concentration between albumin and IL-6 is thus ~10^{10}. This range covers the proteins that are known and considered to be useful as biomarkers but ignores those to be discovered in the future at even lower concentrations. Characterization of the plasma proteome, therefore, poses many challenges. The aim is to create a reliable human blood plasma reference base as a basis for future biomarker discoveries. Many of the established biomarkers were detected by immunoassays and not proteomics, where currently technology is limited to a dynamic range of 10^3–10^4. A number of strategies have been used to overcome the problems and some research projects are underway.

Focus of research during the past decade has been on complete analysis of plasma samples to see all differences rather than targeted analysis to measure one or more hypothesis-generated candidates. Complete analysis has the advantage that it enables the direct selection of optimal biomarker proteins at the outset. The number of proteins detectable in plasma has risen to >1000 as reported in various recent studies. Targeted analysis, however, is more meaningful for disease-biomarker association and hybrid approaches have been proposed that combine the multiprotein view of proteomics and the advantages of targeted specific assays and are termed targeted proteomics.

Technologies for Removal of Highly Abundant Proteins in Blood

Identification of rare proteins in blood is often hindered by highly abundant proteins, such as albumin and immunoglobulin, which obscure less plentiful molecules. As study was done to determine appropriate sample preparation of proteomic

samples in rodent models by examining blood samples (including both serum and plasma) for high abundant protein removal techniques for subsequent gel-based proteomic experiments (Haudenschild et al. 2014). Four methods of albumin removal were assessed: antibody-based affinity chromatography (MARS), Cibacron® Blue-based affinity depletion (SwellGel® Blue Albumin Removal Kit), protein-based affinity depletion (ProteaPrep Albumin Depletion Kit) and TCA/acetone precipitation. Although all four approaches can effectively remove high abundant proteins, antibody-based affinity chromatography was found to be superior to the other three methods.

Multiple Affinity Removal System (Agilent Technologies), an immunoaffinity column, which comprises antibodies to the six most abundant proteins found in human blood is a solution to this problem. By merely running a sample over the matrix, one can specifically remove all six proteins at once, unveiling lower-abundance species that may represent new biomarkers for disease diagnosis and therapy. The process removes about 85% of the total protein mass. The multiple affinity removal system works with blood, CSF, and urine, all of which contain the same major proteins. Blood serum is the favored source for investigators interested in large-scale proteomics; because it is has the most proteins. However, so far only ~500 of the 30,000 proteins in serum have been identified. Removal of albumin and the other 5 major proteins will enable further digging into the proteome.

VIVAPURE Anti-HSA/IgG Kit (Sartorius AG) provides both a highly specific, antibody fragment-based system for albumin removal and a protein G coupled system for IgG removal. These affinity systems are well established for their excellent sensitivity and specificity. 2D separation of proteins from depleted serum showed better resolution of existing spots and revealed an additional 1000 proteins that were not visible in gels of the untreated sample. A convenient, easy-to-use spin column format needing only a lab centrifuge allows parallel processing of different samples. Detailed studies with the new system have shown it to be a highly useful tool for the detection of new biomarkers in blood, synovial fluid, CSF or other protein samples that would otherwise be affected by excess albumin and IgG.

In 2009, a consortium of European companies and universities were awarded €3 million ($3.8 million) under the European Commission's 7th Framework Program to develop a plasma biomarker discovery platform. The 3-year project, called Proactive, was conducted by a consortium consisting of Innova Biosciences, Copenhagen University in Denmark, Fujirebio Diagnostics, Uppsala University in Sweden, and Integromics. The goal of the initiative was to develop methods and reagents for multiplexed proximity ligation assays for detecting low-abundance proteins in plasma, along with statistical methods and data management tools. The participants plan to carry out a series of pilot biomarker projects focused on colorectal cancer detection in blood samples. The participants plan to develop a platform that can analyze 100 samples per week. The platform will be able to detect 180 putative protein biomarkers using four microliters of plasma.

3D Structure of CD38 as a Biomarker

Human CD38 is a multifunctional protein involved in diverse functions. As an enzyme, it is responsible for the synthesis of two Ca^{2+} messengers, cADPR and NAADP; as an antigen, it is involved in regulating cell adhesion, differentiation, and proliferation. Besides, CD38 is a biomarker of progression of HIV-1 infection and a negative prognostic marker of B-CLL. 3D crystal structure CD38 has been determined, which may lead to important discoveries about how cells release calcium. The findings also may offer insights into mechanisms involved in certain diseases, ranging from leukemia to diabetes and HIV-AIDS. For example, CD38 interrupts an interaction between the AIDS virus and its point of entry into cells – a protein receptor called CD4. CD38's 3-D structure reveals a peptide may play a role in interrupting the interface between CD4 and HIV-AIDS. Levels of the protein rise for unknown reasons during illness, making human CD38 a marker for these diseases.

BD™ Free Flow Electrophoresis System

BD™ Free Flow Electrophoresis (FFE) System (BD Biosciences) is a novel separation concept that enables greater penetration of the proteome via separation of a wide variety of charged or chargeable analytes, ranging from small molecules to cells. Unlike traditional electrophoretic or chromatographic techniques, the method provides continuous electrophoretic separation, in the absence of solid phase interactions, providing the simultaneous benefits of reproducibility, speed, a high resolution fractionation gradient, and high recovery. Laboratory results demonstrate an improvement in the resolution on 2D PAGE by more than a factor of five, when compared to unfractionated samples. Additionally, digested fractions analyzed by LC-MS/MS yield hundreds of protein identifications per fraction. A further complementary benefit of this novel technology is the ability to rapidly and efficiently deplete albumin from human plasma during the fractionation process. Additionally, the FFE methods developed are appropriate for processing large numbers of samples, allowing high sample throughput without sacrificing resolution or protein concentration dynamic range, which should improve identification of novel protein biomarkers in plasma.

Isotope Tags for Relative and Absolute Quantification

Life Technologies Corp has developed Isotope tags for relative and absolute quantification (iTRAQ™), which is potentially more cost-effective because up to four samples can be screened simultaneously with four isotopically distinguishable reagents. The iTRAQ method utilizes isobaric tags containing both reporter and balancer groups. The reporter is quantitatively cleaved during collision-induced

dissociation (CID) to yield an isotope series representing the quantity of a single peptide of known mass from each of up to four different samples. This quantification group (the reporter) is 'balanced' by a second group (the balancer) depleted of the same stable isotopes, which maintains each isotopic tag at exactly the same mass. Since the peptide remains attached to the isobaric tags until CID is conducted, the peptide is simultaneously fragmented for sequence identification.

The current generation of iTRAQ reagents labels lysine residues and the N termini of peptides, meaning that most peptides are multiply labeled (as with GIST). Therefore, iTRAQ suffers the same peptide overabundance problem and must be coupled with one or more dimensions of chromatographic or electrophoretic separation before MS analysis to limit the number of isobaric tagged peptides in the first MS dimension. The advantage of iTRAQ over these methods is that the label is cleaved in the tandem MS before quantification.

Because differences in peptide levels can only be determined after tandem MS, the first MS dimension cannot be used to pre-screen peptides for differential expression before tandem MS identity determination. Therefore, each and every peptide must be subjected to tandem MS analysis, making iTRAQ both time-consuming and sample-intensive for biomarker discovery applications. Furthermore, any untagged isobaric chemical noise may confound tandem-MS sequencing of the iTRAQ labeled peptides.

Another issue with this method is the problem of protein variants. Any variant of the peptide of interest will not be isobaric with the same tagged peptides from control samples. Such non-isobaric peptides can be detected by their absence, but may be falsely interpreted as down-regulation of the parent protein. Furthermore, such peptides may be isobaric with other peptides, confounding the interpretation of expression levels or sequences of other peptides. However, in target validation, patient profiling or toxicological screening applications, where the masses of the peptides are known, iTRAQ™ is potentially very cost-effective.

Plasma Protein Microparticles as Biomarkers

Although current proteomic technologies enable detection and analysis of extremely small amounts of proteins (picomole to attomole level), it is difficult to detect and quantify proteins present at 2- to 3-orders of magnitude lower than the more abundant proteins. Microparticles are subcellular particles varying in size from 50 nm to 1 μm that are released by essentially all cells upon activation. They have a variety of important physiological and pathophysiological functions including role as the main carrier of tissue factor in the blood and participating in intercellular communications. In the plasma of healthy individuals, over 90% originate from platelets. Other sources of microparticles are cancer cells, endothelial cells including those form newly formed angiogenic vessels, leukocytes and smooth muscle cells. Although this subproteome makes up less than 0.01% of the total plasma proteome, it is rich in proteins altered under a variety of pathological conditions. The proteome of microparticles isolated from the plasma of healthy individuals is slightly but significantly different from that of platelet microparticles.

The total number and the cellular origin of these microparticles is altered in a wide variety of pathological conditions including cardiovascular diseases. The protein composition of microparticles differs according to the disease. Normal biological fluids used for biomarker discovery, such as plasma or urine, contain a small number of proteins present at much higher amounts than the remaining proteins. For example, in the plasma, albumin and immunoglobulins are present at milligrams per milliliter, while proteins of interest for biomarker discovery may be present at micrograms to picograms per ml. Microparticle subproteome is being investigated as a potential source of biomarkers. MicroParticle Proteomics is developing microparticle-based biomarker screening and diagnostic tests for cardiovascular diseases.

Proteome Partitioning

Protein depletion has been used for some years to remove most of the albumin and/or IgG from biofluids such as plasma and serum prior to analysis, but it is clear that this alone is insufficient to enable progress to be made in biomarker discovery. The presence of highly abundant proteins significantly complicates the discovery process by masking the presence and limiting the detection of low abundance species. ProteomeLab IgY partitioning (Beckman Coulter) addresses this issue by reversibly capturing 12 of the more abundant proteins from human biofluids such as plasma and serum, yielding an enriched pool of low abundance proteins for further study. The captured proteins can also be easily recovered for investigation if required – hence the term partitioning rather then depletion. IgY-12 selectively partitions the 12 highly abundant proteins and the partitioned fractions can be taken to the next stage of the discovery process, such as multidimensional fractionation using the ProteomeLab PF 2D system or profiling using 2D PAGE.

Stable Isotope Tagging Methods

Stable isotope tagging methods provide a useful means of determining the relative expression level of individual proteins between samples in a mass spectrometer with high precision. Because two or more samples tagged with different numbers of stable isotopes can be mixed before any processing steps, sample-to-sample recovery differences are eliminated. Mass spectrometry also allows post-translational modifications, splice variations and mutations (often unnoticed in immunoassays) to be detected and identified, increasing the clinical relevance of the assay and avoiding the issues of non-specific binding and cross-reactivity observed in immunoassays. Several stable isotope tagging methods have evolved over the last decade and are undergoing development in a number of different areas, such as; mass spectrometric instrumentation, peptide identification algorithms and bioinformatic computational data analysis. Improved methods enable quantitative measurement of relative or absolute protein amounts, which is essential for gaining insights into their functions and dynamics in biological systems. Several different strategies

involving stable isotopes labels, label-free statistical assessment approaches and absolute quantification methods are possible, each with specific strengths and weaknesses.

Baiting and affinity pre-enrichment strategies, which overcome the dynamic range and sample complexity issues of global proteomic strategies, are very difficult to couple to MS. This is due to the fact that it is nearly impossible to sort target peptides from those of the bait since there will be many cases of isobaric peptides. Isotope-differentiated binding energy shift tags (IDBEST) has been developed by Target Discovery Inc as a tagging strategy that enables such pre-enrichment of specific proteins or protein classes as the resulting tagged peptides are distinguishable from those of the bait by a mass defect shift of ~0.1 atomic mass units. The special characteristics of these tags allow: resolution of tagged peptides from untagged peptides through incorporation of a mass defect element; high-precision quantitation of up- and downregulation by using stable isotope versions of the same tag; and potential analysis of protein isoforms through more complete peptide coverage from the proteins of interest.

Inductively coupled plasma MS (ICP-MS), still widely recognized as elemental detector, has emerged as a complementary technique to previous methods. The new application of ICP-MS is targeting the fast growing field of proteomics related research, enabling absolute protein quantification using suitable elemental based tags (Chahrour et al. 2015).

Technology to Measure Both the Identity and Size of the Biomarker

While SELDI-TOF platforms provided MS approaches that could generate size and identity information, the inability of surface-based chromatography to provide a strategy for low-abundance analytes necessitates the development of new approaches of fragment-based analyte detection. One ideal format would be high-throughput MS technology coupled with true affinity chromatography whereby larger quantities of body fluids could be queried over a flow-through high-capacity surface. Therefore, the future of peptide-based diagnostics will require the invention and adoption of wholly new technologies that rapidly read both the identity and the exact size of the molecule. Immuno-MS provides a means to do this. Using this technology, a microaffinity antibody column, perhaps in a multiplexed microwell format, is first used to capture all species of molecules that contain the antibody recognition site, regardless of size. MS analysis of eluted peptides provides an extremely accurate mass determination of the entire population of captured peptides. Thus, in only two steps, immuno-MS can rapidly tabulate a panel of peptide fragments derived from a known parent molecule.

Conventional immunoassay and newer multiplexed technologies such as antibody arrays and suspension bead arrays cannot measure panels of peptide analytes that carry their diagnostic information based in two dimensions of both size and identity. PerkinElmer is working with Center for Applied Proteomics and Molecular Medicine at George Mason University (Fairfax, VA) to develop an immunoassay-based

application using a mass spectrometer as the detector rather than a fluorescence detector. The approach could provide a solution to the challenge of multiplexed fragment-based analyte measurements. Other types of configured formats, such as plasmon resonance-based affinity mass spectrometry, may also be successful in translating mass spectrometry into clinic applications.

Selected Reaction Monitoring MS

Selected reaction monitoring (SRM) MS was used to determine the relative quantitation of 31 plasma proteins commonly observed in biomarker studies over various time courses (Randall et al. 2012). Estimates of between-subject and within-subject biological variances determined by SRM were calculated for each protein. Replicate analysis demonstrated the high precision of SRM assays of plasma proteins. Statistical analysis indicated that none of the measured proteins exhibited significant temporal fluctuations over either time course. Overall, time-based intra-individual quantitative variation of plasma protein levels was considerably lower than biological variation occurring between individual volunteers. This study is the first to show robust temporal stability of the plasma proteome in healthy individuals using SRM-based peptide quantitation. This is important as it provides a strong basis for reliable detection of disease/treatment related changes of these plasma proteins and others using SRM.

Targeted MS for Verification of Biomarkers

High-throughput technologies can now identify hundreds of candidate protein biomarkers for any disease with relative ease. However, because there are no assays for the majority of proteins and de novo immunoassay development is prohibitively expensive, few candidate biomarkers are tested in clinical studies. Targeted MS is capable of quantifying biomarker candidates at concentration ranges where biomarkers are expected in plasma (i.e. at the ng/ml level). A published workflow enables fast and definitive generation of targeted MS-based assays for most biomarker candidate proteins (Hüttenhain et al. 2009). These assays are stored in publicly accessible databases and have the potential to greatly impact the throughput of biomarker verification studies. Analytical performance of a biomarker identification pipeline based on targeted MS may be sufficient for data-dependent prioritization of candidate biomarkers, de novo development of assays and multiplexed biomarker verification (Whiteaker et al. 2011).

Biomarkers in the Urinary Proteome

Plasma membrane proteins are likely present in urine by secretion in exosomes. Urine is a desirable material for the diagnosis and classification of diseases due to the convenience of collection in large amounts. However the urinary proteome

catalogs currently being generated have limitations in their depth and confidence of identification. Methods involving a linear ion trap – Fourier transform (LTQ-FT) and a linear ion trap – orbitrap (LTQ-Orbitrap) MS have been developed for the in-depth characterization of body fluids and applied to the analysis of the human urinary proteome. More than 1500 proteins were identified in the urine obtained from ten healthy donors, and nearly half of the these were membrane proteins. This analysis provides a high confidence set of proteins present in human urinary proteome and provides a useful reference for comparing datasets obtained with different methods. The urinary proteome is unexpectedly complex and may prove useful in biomarker discovery in the future.

Peptides as Biomarkers of Disease

Bodily fluids contain a vast array of low-molecular-weight (LMW) peptides generally produced from larger precursor proteins. Mapping these LMW fragments back to the parent molecules has revealed evidence of a complex protein processing system. Furthermore, changes in the normal protein/peptide repertoire reflect pathological processes that correlate with disease susceptibility, disease state, or both. For example, the progressive inflammation and destruction of the salivary glands in patients with oral cancer or Sjögren's Syndrome (an autoimmune disease) will lead to specific protein and peptide alterations in saliva. Thus, identification of disease-associated differences in enzyme composition and activity will lead to a better understanding of the molecular basis of diseases and will facilitate the identification of biomarkers in patients that may assist in screening, diagnosing, and monitoring the disease state.

The low molecular weight region of the serum peptidome contains protein fragments derived from two sources: (1) high-abundance endogenous circulating proteins; and (2) cell and tissue proteins. MS-based profiling has indicated that the peptidome may reflect biological events and contain diagnostic biomarkers. Recent studies have established distinctive serum polypeptide patterns through MS that reportedly correlate with clinically relevant outcomes. Wider acceptance of these signatures as valid biomarkers for disease may follow sequence characterization of the components and elucidation of the mechanisms by which they are generated.

Peptidomics technologies provide new opportunities for the detection of low-molecular-weight proteome biomarkers (peptides) in body fluids. Improvements in peptidomics research are based on separation of peptides and/or proteins by their physicochemical properties in combination with mass spectrometric detection, identification and sophisticated bioinformatics tools for data analysis. These provide an opportunity to discover novel biomarkers for diagnosis and management of disease.

An example of the application of Peptidomics® Technologies (BioVision AG) is the study of plasma samples of diabetic patients for biomarkers before and after oral glucose challenge and validation by immunoassays. Peptidomics analysis enabled

display of >1500 circulating peptides from 1 mL EDTA-plasma and excellent sensitivity ranges reaching ~100 pmol/L (pg/mL). Quantitative changes were detected in the picomolar range. Known diagnostic biomarkers were found in a Differential Peptide Display and validated by ELISA-measurement. Unknown, putative biomarkers could have been discovered and inter-individual differences were demonstrated.

Analysis of Peptides in Bodily Fluids

Current methods for detecting enzyme abundance and activity are limited because: (1) they lack the requisite sensitivity to detect the low-level activities that are typically present in bodily fluids; (2) they rely on prior knowledge of the identity of the enzymes present in the sample. Thus, new methods are being explored for better detection of enzymes that would lead to improved diagnostic and/or prognostic assays for various diseases.

Researchers at University of California (San Francisco, CA) have developed a novel method to characterize enzyme activity that can be used to diagnose diseases or monitor stability of biological samples. The method involves MS analysis of alterations in the endogenous peptide repertoire and/or custom exogenous probes. Advantages of this method are:

- Inexpensive and rapid proteinase activity assay.
- Direct tool to differentiate between post-translational vs. transcriptional regulation of peptide production.
- Comprehensive, non-biased analyses that do not require prior knowledge of proteinase identity.
- Improved sensitivity with amplified detection of enzymatic activity pointing to the identity of the enzyme species in situation where the abundance of these molecules is below the detection limit of other methods.
- These biomarkers can form the basis of diagnostic test for human diseases that have proteolytic components (e.g., autoimmune diseases, cancer).
- N-terminal peptide isolation from human plasma
- Problems such as dynamic fluctuations in protein concentration of protein biomarkers in human plasma is a drawback for exploiting this resource. A method to label and isolate N-terminal peptides from human plasma and serum has been described, which dramatically reduces the complexity of the sample by eliminating internal peptides (Wildes and Wells 2010). This approach is highly suited for studying natural proteolysis in plasma and serum. Internal cleavages were found in plasma proteins created by endo- and exopeptidases, providing information about the activities of proteolytic enzymes in blood, which may be correlated with disease states. Other findings included signatures of signal peptide cleavage, coagulation and complement activation, and other known proteolytic processes. Finally, substrates could be identified from specific proteases by exogenous addition of the protease combined with N-terminal isolation and

quantitative mass spectrometry. In this way proteins cleaved in human plasma by membrane-type serine protease 1, an enzyme linked to cancer progression, were identified. These studies demonstrate the usefulness of direct N-terminal labeling by subtiligase to identify and characterize endogenous and exogenous proteolysis in human plasma and serum.

Antibody Biomarker Discovery via Evolution of Peptides

To enable discovery of serum antibodies indicative of disease and simultaneously develop reagents suitable for diagnosis, in vitro directed evolution has been applied to identify consensus peptides recognized by patients' serum antibodies (Ballew et al. 2013). Bacterial cell-displayed peptide libraries were quantitatively screened for binders to serum antibodies from patients with celiac disease (CD), using cell-sorting instrumentation to identify two distinct consensus epitope families specific to CD patients (PEQ and $^{E}/_{D}$xFV$^{Y}/_{F}$Q). Evolution of the $^{E}/_{D}$xFV$^{Y}/_{F}$Q consensus epitope identified a celiac-specific epitope, distinct from the two CD hallmark antigens tissue transglutaminase-2 and deamidated gliadin, exhibiting 71% sensitivity and 99% specificity. Expansion of the first-generation PEQ consensus epitope via in vitro evolution yielded octapeptides QPEQAFPE and PFPEQxFP that identified ω- and γ-gliadins, and their deamidated forms, as immunodominant B cell epitopes in wheat and related cereal proteins. The evolved octapeptides, but not first generation peptides, discriminated one-way blinded CD and non-CD sera with exceptional accuracy, yielding 100% sensitivity and 98% specificity. Because this method does not require prior knowledge of pathobiology, it may be broadly useful for de novo discovery of antibody biomarkers and reagents for their detection. It may enable development of effective diagnostic tests for other medical conditions where such tests are lacking and the identification of environmental factors involved in disease.

Serum Peptidome Patterns

The peptidome information is archived in at least three dimensions: (1) the identity of the peptide (e.g., the peptide sequence or parent protein from which it was derived); (2) the quantity of the peptide itself; and (3) the state of the modified form (fragment size and cleavage ends, posttranslational glycosylation sites, etc). Peptide fragments are developed by the disease system and embody an integrated record of the system. Thus, taking a systems biology approach to measure panels of peptidome markers can potentially overcome the failures of previous biomarkers to achieve adequate clinical sensitivity and specificity.

A study using a highly optimized peptide extraction and MALDI-TOF MS-based approach has shown that a limited subset of serum peptides (a signature) can provide accurate class discrimination between patients with three types of solid tumors and controls without cancer (Villanueva et al. 2006). This small but robust set of

marker peptides has enabled a highly accurate class prediction for an external validation set of prostate cancer samples. This study provides a direct link between peptide marker profiles of disease and differential protease activity, and the patterns described may have clinical utility as surrogate markers for detection and classification of cancer. These findings also have important implications for future peptide biomarker discovery efforts.

SISCAPA Method for Quantitating Proteins and Peptides in Plasma

Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) is a proteomics platform aimed at biomarker quantification. It combines immunoenrichment with selected reaction monitoring (SRM) MS. Anti-peptide antibodies immobilized on 100 nL nanoaffinity columns are used to enrich specific peptides along with spiked stable-isotope-labeled internal standards of the same sequence. Upon elution from the anti-peptide antibody supports, electrospray MS is used to quantitate the peptides (natural and labeled). SISCAPA is thus limited to sequence-defined (predetermined) analytes, but offers the possibility of greatly increased sensitivity (by removing unwanted peptides from the set delivered to the MS). This technique is being used to develop a new proteomics assay for diagnosing Alzheimer's disease. Using this assay, it was shown that FGF15 circulates in plasma in a circadian rhythm-dependent manner at concentrations that activate its receptor. Consistent with the proposed endocrine role for FGF15 in liver, mice lacking hepatocyte expression of the obligate FGF15 co-receptor, β-Klotho, have increased bile acid synthesis and reduced glycogen storage despite having supraphysiological plasma FGF15 concentrations. Collectively, these data demonstrate that FGF15 functions as a hormone and highlight the utility of SISCAPA-SRM as a sensitive assay for detecting low-abundance proteins in plasma (Katafuchi et al. 2015).

Comparison of Proteomic Profiling Technologies for Discovery of Biomarkers

A comparison of proteomic profiling technologies for discovery of biomarkers is shown in Table 2.2.

Verification for Interlaboratory Reproducibility of Protein Biomarkers

Discovery proteomics often results in a list of tens or even hundreds of potential biomarkers, but because analyzing each biomarker can take up to several weeks and the sample numbers are low, the false discovery rates tend to run high. This is not

Table 2.2 Comparison of proteomic profiling technologies for discovery of biomarkers

Approaches	Ability to detect isoforms	Precision in relative quantitation	Ability to detect low abundance proteins	High speed, low cost and simplicity
Optical methods (e.g., ELISA, SPR)	No	Yes	Yes	Yes
MudPIT	Yes	No	No	No
ProteinChip®	Yes	No	No	Yes
ICAT	Yes	Yes	No	No
ITRAQ™ (Applied Biosystems)	Yes	Yes	No	No
Isonostics™ (Target Discovery Inc)	Yes	Yes	Yes	Yes

Modified from Hall and Schneider (2004)
Abbreviations: *SPR* surface plasma resonance, *MudPIT* multidimensional protein identification technology, *ELISA* enzyme-linked immunosorbent assay, *ICAT* isotope-coded affinity tag

necessarily because of technical variability but rather is the consequence of biological variability in the samples.

Verification of candidate biomarkers relies upon specific, quantitative assays optimized for selective detection of target proteins, and is increasingly viewed as a critical step in the discovery pipeline that bridges unbiased biomarker discovery to preclinical validation. Such technology has been limited by the availability of well-characterized antibodies, a well-known problem in protein research. Developing high-quality immunoassays also is costly and time-consuming. Although individual laboratories have demonstrated that multiple reaction monitoring (MRM) coupled with isotope dilution mass spectrometry can quantify candidate protein biomarkers in plasma, reproducibility and transferability of these assays between laboratories have not been demonstrated.

A multilaboratory study has assess reproducibility, recovery, linear dynamic range and limits of detection and quantification of multiplexed, MRM-based assays, conducted by NCI-CPTAC (Addona et al. 2011). Using common materials and standardized protocols, it was shown that these assays can be highly reproducible within and across laboratories and instrument platforms, and are sensitive to low microgram per milliliter protein concentrations in unfractionated plasma. The study has provided data and benchmarks against which individual laboratories can compare their performance and evaluate new technologies for biomarker verification in plasma. Such methods, combined with protein- and peptide-enrichment strategies, are able to hit target values for limits of quantitation that are in the very bottom of the nanogram per milliliter range for proteins in blood, where many biomarkers of clinical utility are located.

Significance of Similar Protein Biomarkers in Different Tissues

The specificity of proteins identified by proteomics as biomarkers for defined conditions or as components of biological processes and pathways is crucial. Usually 2DGE analysis of proteins expressed in two different but related samples, such as healthy and diseased organs leads to the assumption that those proteins expressed at different levels in the two organs are linked to the disease in some way and can be used as biomarkers for it. But this simple idea could well be wrong. Protein biomarkers discovered by one research group are often not confirmed by other groups. This may be due to laboratory errors but another explanation is that many of the differently expressed proteins are not actually specific for the disease or condition being investigated. Even if the researchers are correctly identifying differently expressed proteins in the two samples, the difference may have nothing to do with disease.

A study reported that reading of several 2DGE-based articles featuring lists of differentially expressed proteins reveals that the same proteins seem to predominate regardless of the experiment, tissue or species (Petrak et al. 2008). The most frequently identified protein was a highly abundant glycolytic enzyme enolase 1, differentially expressed in nearly every third experiment on both human and rodent tissues. Heat-shock protein 27 (HSP27) and heat-shock protein 60 (HSP60) were differentially expressed in about 30% of human and rodent samples, respectively. Considering protein families as units, keratins and peroxiredoxins are the most frequently identified molecules, with at least one member of the group being differentially expressed in about 40% of all experiments. The authors wondered if these commonly observed changes represent common cellular stress responses or are a reflection of the technical limitations of 2DGE.

In another study, differentially expressed proteins from comparative proteomic studies identified by 2DGE followed by MS, especially with MALDI technique, were critically reviewed (Wang et al. 2009). Based on 66 of those studies, a list of 44 proteins was presented as generally detected proteins regardless of species, in vivo or in vitro conditions, tissues and organs, and experimental objective. Similarly, a list of 28 generally detected protein families was presented. The enriched functions linked to these generally detected proteins reveal that there are some common biological features beyond the technical limitations. Cellular stress response can be the universal reason as to why these proteins are generally expressed differentially. Using those proteins as biomarkers for cellular processes other than stress response should be done with caution. Such disease biomarkers would be merely pinpointing stressed out cells with no useful application. In future proteomic studies more profound approaches should be applied to look beyond these proteins to find specific biomarkers.

Glycomic Technologies

Mass spectrometry (MS), in combination with modern separation methodologies, is one of the most powerful and versatile techniques for the structural analysis of glycans (oligosaccharides). NMR spectroscopy and computer graphic modeling can be used to display the dynamic structure of protein glycosylation. These methods can be used for intermolecular and intramolecular protein-oligosaccharide interactions and complement X-ray crystallography.

Proteome Systems has applied specific protocols and bioinformatics in its proteomics platform, ProteomIQ, to glycomics. These technologies cover all areas of glycan analysis, from glycoprotein sample preparation to glycan structural analysis at the level of analyzing glycoprotein isoforms separated by gel electrophoresis. Glycan fragmentation MS data is interpreted by specific bioinformatic software integrated with the company's glycan database, GlycoSuite DB, to automatically generate the corresponding oligosaccharide structures. This technology bridges the gap between proteomics and glycomics.

Ezose Sciences offers comprehensive glycan analytical services using its proprietary GlycanMap® platform technology that combines an automated glycan sample processor with high-throughput MALDI-TOF-MS or research of glycan-related biomarkers.

Cellular Glycomics for Discovery of Cellular Biomarkers

Although many of the frequently used pluripotency biomarkers are glycoconjugates, a glycoconjugate-based exploration of novel cellular biomarkers has proven technically difficult. A unique approach has been reported for the systematic overview of all major classes of oligosaccharides in the cellular glycome (Fujitani et al. 2013). This method enabled MS-based structurally intensive analyses, both qualitative and quantitative, of cellular N- and O-linked glycans derived from glycoproteins, glycosaminoglycans, and glycosphingolipids, as well as free oligosaccharides of human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs). Cellular total glycomes were found to be highly cell specific. Structures of glycans of all classes specifically observed in hESCs and hiPSCs tended to be immature in general, suggesting the presence of stem cell-specific glycosylation spectra. Analysis revealed the high similarity of the total cellular glycome between hESCs and hiPSCs, although it was suggested that hESCs are more homogeneous than hiPSCs from a glycomic standpoint. This study enabled identification of known pluripotency biomarkers such as SSEA-3, -4, and -5 and Tra-1-60/81, as well as a panel of glycans specifically expressed by hESCs and hiPSCs.

Metabolomic Technologies

Within the last few years, metabolomics has developed into a technology that complements proteomics and transcriptomics. In combination with techniques for functional analysis of genes, it is hoped that a holistic picture of metabolism can be formed. In addition to the genome analysis and proteome analyses, the exhaustive analysis of metabolites is important for a comprehensive understanding of cellular functions because the dynamic behavior of metabolites cannot be predicted without information regarding metabolome.

In view of the chemical and physical diversity of small biological molecules, the challenge remains of developing protocols to gather the whole ‘metabolome'. No single technique is suitable for the analysis of different types of molecules, which is why a mixture of techniques has to be used. In the field of metabolomics, the general estimations of the size and the dynamic range of a species-specific metabolome are at a preliminary stage. Metabolic fingerprinting and metabonomics with high sample throughput but decreased dynamic range and the deconvolution of individual components achieve a global view of the in vivo dynamics of metabolic networks. The technologies used include NMR, direct infusion mass spectrometry, and/or infrared spectroscopy. Gas chromatography (GC)–MS and LC–MS technology achieve a lower sample throughput but provide unassailable identification and quantitation of individual compounds in a complex samples.

However, it is important to note that each type of technology exhibits a bias towards certain compound classes, mostly due to ionization techniques, chromatography and detector capabilities. GC-MS has evolved as an imperative technology for metabolomics due to its comprehensiveness and sensitivity. The coupling of GC to time-of-flight (TOF) mass analyzers is an emerging technology. High scan rates provide accurate peak deconvolution of complex samples. GC-TOF-MS capabilities provide an improvement over conventional GC-MS analysis in the analysis of ultracomplex samples, which is particularly important for the metabolomics approach. Ultracomplex samples contain hundreds of co-eluting compounds that vary in abundance by several orders of magnitude. Thus, accurate mass spectral deconvolution and a broad linear dynamic range represent indispensable prerequisites for high quality spectra and peak shapes. Modern GC-TOF-MS applications and incorporated mass spectral deconvolution algorithms fulfill these requirements. The advantages of metabolomics technologies are:

- Ability to analyze all bodily fluids such as blood, CSF and urine as well as cultured or isolated cells and biopsy material.
- High throughput capability enabling simultaneous monitoring of biological samples
- Analysis of multiple pathways and arrays of metabolites simultaneously from microliter sample quantities.

In 2015, the Canadian Government gave a grant of C$3 million (US$2.4 million) towards instruments to help establish the Metabolomics Technology Demonstration Centre, in partnership with Genome Alberta and the University of Alberta. The instruments, a 700 MHz NMR machine and a quadrupole TOF MS, will assist companies in the metabolomics sector translate biomarker-based tests from the lab into the clinic. Companies will test, validate, and assemble prototypes using the University of Alberta Metabolomics Innovation Centre's biomarker panels, to create more accurate and less invasive medical tests.

Genome-Wide Association Studies for Identification of Metabolic Biomarkers

Genome-wide association studies (GWAS) reveal genetic variants that confer increased or decreased susceptibility to such complex diseases such as metabolic disorders, e.g. type 2 diabetes. To be able to use the GWAS in a clinical setting is a complex challenge, yet it is hoped that, in the future, this tool will ultimately enable the development of pharmaceutical options that are capable of targeting the cause of metabolic disorders, not just the symptoms themselves (Pattin and Moore 2010).

Genetic Influences on Human Blood Metabolites

Genome-wide association scans with high-throughput metabolic profiling provide unprecedented insights into how genetic variation influences metabolism and complex disease. A study has reported the most comprehensive exploration of genetic loci influencing human metabolism so far, comprising 7824 adult individuals from 2 European population studies (Shin et al. 2014). Genome-wide significant associations were found at 145 metabolic loci and their biochemical connectivity was shown with more than 400 metabolites in human blood. The resulting in vivo blueprint of metabolism in human blood was extensively characterize by integrating it with information on gene expression, heritability and overlap with known loci for complex disorders, inborn errors of metabolism and pharmacological targets. The authors further developed a database and web-based resources for data mining and results visualization. These findings provide new insights into the role of inherited variation in blood metabolic diversity and identify potential new opportunities for drug development and for understanding disease.

Lipid Profiling

Single biomarkers, such as plasma cholesterol or triglycerides are used in modern medicine to assess the risk of certain diseases, butsuch assessments overlook the inherent complexity of metabolic disorders involving hundreds of biochemical

processes. Assessing the full breadth of lipid metabolism is what drives the field of lipomic profiling. However, unlike the other "-omic" technologies, in which only a small portion of the genes or proteins is known, lipid metabolic pathways are well characterized. Another limitation of "-omics" technologies is that they produce so many false positive results that it is difficult to be sure that findings are valid. Metabolomics is not immune to this problem but, when practiced effectively, the technology can reliably produce knowledge to aid in decision making. Focused metabolomics platforms, which restrict their target analytes to those measured well by the technology, can produce data with properties that maximize sensitivity and minimize the false discovery problem. TrueMass® (Lipomic Technologies) analysis produces lipomic profiles – comprehensive and quantitative lipid metabolite profiles of biological samples. TrueMass® measures hundreds of lipid metabolites from a small quantity of tissue or serum sample. Because the resulting data are quantitative, TrueMass data can be seamlessly integrated with pre-existing or future databases.

Data-dependent acquisition of MS/MS spectra from lipid precursors enables emulation of the simultaneous acquisition of an unlimited number of precursor and neutral loss scans in a single analysis. This approach takes full advantage of rich fragment patterns in tandem mass spectra of lipids and enables their profiling by complex scans, in which masses of several fragment ions are considered within a single logical framework. No separation of lipids is required, and the accuracy of identification and quantification is not compromised, compared to conventional precursor and neutral loss scanning.

Mass Spectrometry for Discovery of Metabolic Biomarkers in Plasma

MS-based kits are being developed for discovery of metabolic biomarkers. The best known of these is AbsoluteIDQ™ Kit (Biocrates Life Sciences), which works with Applied Biosystems API 4000 and API 4000 QTRAP ® Mass Spectrometer. It enables accurate identification and quantification of >160 metabolites in >4 compound classes from clinical and preclinical plasma samples within a few minutes. This is the first integrated technology platform (metabolomics, genomics and proteomics), which automates proprietary standardized pre-analytical and analytical steps for metabolic analysis using bioinformatics. It provides a sample base with access to clinical data for discovery, validation and evaluation of new biomarkers and drug targets.

ROA Technologies' Mass Spectrometry Metabolite Library of Standards (MSMLS) and its corresponding software, MSMLSDiscovery are marketed by Sigma-Aldrich to provide retention times and spectra for key metabolic compounds. It also helps to optimize MS analytical protocols and to qualify and quantify MS sensitivity and limit of detection.

Currently, only 1.8% of spectra in an untargeted metabolomics experiment can be annotated. To link the large volume of known chemical structures to the data obtained with MS remains a challenge because reference spectra are not available in MS databases. This means that the vast majority of information collected by metabolomics is "dark matter," chemical signatures that remain uncharacterized. Much of the chemical dark matter may include known structures, but they remain undiscovered. The only way to overcome this challenge is through the development of computational solutions. A computational tool called CSI (compound structure identification):FingerID is designed to aid in the annotation of chemistries that can be observed by MS (Dührkop et al. 2015). CSI:FingerID uses fragmentation trees to connect tandem MS (MS/MS) data to chemical structures found in public chemistry databases. Tools such as this can enable metabolomics with MS to become as commonly used and scientifically productive as sequencing technologies are in the field of genomics.

Role of Metabolomics in Biomarker Identification and Pattern Recognition

Metabolomics research has increased significantly over recent years due to advances in analytical measurement technology and the advances in pattern recognition software enabling one to visualize changes in levels of hundreds or even thousands of chemicals simultaneously. Multivariate metabolomic and proteomic data and time-series measurements can be combined to reveal protein-metabolite correlations. Different methods of multivariate statistical analysis can be explored for the interpretation of these data. The discrimination of the samples enables the identification of novel components. These components are interpretable as inherent biological characteristics.

Biomarkers that are responsible for these different biological characteristics can easily be classified because of the optimized separation using independent components analysis and an integrated metabolite-protein dataset. Evidently, this kind of analysis depends strongly on the comprehensiveness and accuracy of the profiling method, in this case metabolite and protein detection. Assuming that the techniques will improve, more proteins and metabolites can be identified and accurately quantified, the integrated analysis will have great promise.

Urinary Profiling by Capillary Electrophoresis

Metabolomic approaches have become particularly important for discovery of biomarkers in urine. The analytical technology for urinary profiling must be efficient, sensitive and offer high resolution. Until recently these demands were commonly

met by HPLC-MS, GC-MS and NMR. The analytical armory for urinary profiling includes cyclodextrin-modified micellar electrokinetic capillary chromatography (CD-MECC), which enables highly cost-effective, rapid and efficient profiling with minimal sample volume and preparation requirements. The CD-MECC profiles typically show separation for >80 urinary metabolites. These profiles have been visualized using novel advanced pattern recognition tools. Visualization of pattern changes has been achieved through development of the novel ACE (Automated Comparison of Electropherograms) software which not only removes errors due to baseline shifts but also allows for rapid reporting of semiquantitative profile differences. The method has been applied in investigation of biomarkers characteristic of alcoholics or Down's syndrome persons.

Validation of Biomarkers in Large-Scale Human Metabolomics Studies

Large scale metabolomics studies require a careful analytical and statistical protocol. Methods combining several well-established statistical tools have been developed for processing this large data set in order to detect small differences in metabolic profiles in combination with a large biological variation. These include data preprocessing, data analysis, and validation of statistical models. After several data preprocessing steps, partial least-squares discriminate analysis (PLS-DA) was used for finding biomarkers. To validate the biomarkers statistically, the PLS-DA models are validated by means of a permutation test, biomarker models, and noninformative models. Univariate plots of potential biomarkers are used to obtain insight in up- or down-regulation.

Lipidomics

Lipidomics is the use of high-dimensional lipid analysis technologies that provide researchers with an opportunity to measure lipids on an unprecedented scale. The development of lipidomics has been accelerated by the concepts of systems biology and advances in the following areas:

- Proteomics, particularly ESI/MS. Shotgun lipidomics, based on multi-dimensional MS array analyses after multiplexed sample preparation and intrasource separation, has matured as a technique for the rapid and reproducible global analysis of cellular lipids.
- Recognition of the role of lipids in many common metabolic diseases.
- Recognition that metabolism of lipids is linked to and requires the simultaneous analysis of multiple lipid classes, molecular species and metabolic networks.

At its current stage, this technology enables us to analyze more than 20 lipid classes and thousands of individual lipid molecular species directly from lipid extracts of biologic samples (Gross and Han 2007). Substantial progress has been made in the application of lipidomics in biomarker discovery. Notable are the applications in the following areas:

- Substantial alterations have been found in myocardial cardiolipin content in early stages of diabetes.
- Obesity has been related to increase in the content of lysophosphatidylcholine molecular species independently of genetic influences and is related to insulin resistance.
- Neurolipidomics is important for discovery of biomarkers of diseases of the nervous system as lipids make up half of the human brain in dry weight.

Lipidomics is moving towards more methodical and structured approaches to biomarker identification. Experimental designs focusing on well-defined outcomes have a better chance of producing biologically relevant results and strategies for improving the quality of data analysis.

Disease Biomarkers in Breath

Since ancient times, physicians have known that human breath can provide clues to diagnosis of various diseases, e.g. the sweet, fruity odor of acetone indicates uncontrolled diabetes. Researchers have identified over 1000 different compounds contained in human breath. These molecules have both endogenous and exogenous origins and provide information about physiological processes occurring in the body as well as environment-related ingestion or absorption of contaminants.

Among commonly used exhaled biomarkers, nitric oxide (NO) on exhaled air and some constituents of exhaled breath condensate in volatile or non-volatile form may represent suitable biomarkers (Maniscalco et al. 2009). Nasal, bronchial and alveolar NO could be analyzed separately, with implications in the assessment of systemic disease and endothelial dysfunction. Moreover, the profiles of several exhaled gases have a place in phenotyping diabetic patients and their risk of complications. Accordingly, metabolomics of the airway fluid using exhaled breath condensate is useful for the evaluation of both volatile and non-volatile biomarkers. Reference values are, however, lacking, and the influence of preanalytical variables on the methods requires further studies.

Several methods for trace molecular detection have been applied to the problem of breath analysis. These include optical detection, mass spectrometry, and electronic noses.

Portable Breath Test for Volatile Organic Compounds

Volatile organic compounds (VOCs) are present in normal human breath and can be detected by gas chromatography (GC). Some of these breath VOCs may be biomarkers of disease, but there are technical problem in collecting breath, analyzing low concentrations of VOCs, and distinguishing normal from abnormal findings. Menssana Research Inc has overcome these technical problems and developed a portable breath collection apparatus which can collect breath samples virtually anywhere. It captures breath VOCs onto a small sorbent trap, which is sent to the laboratory for analysis by GC and MS. Each analysis usually identifies more than 200 different VOCs. This breath test has identified a new and comprehensive set of biomarkers of oxidative stress known as the breath methylated alkane contour (BMAC). Changes in the BMAC have revealed distinctive patterns in a number of different diseases each can be identified. This breath test is now being evaluated in several clinical studies, including:

- Lung cancer
- Breast cancer
- Heart transplant rejection
- Kidney disease
- Ischemic heart disease
- Diabetes mellitus
- Pulmonary tuberculosis

Detection of Breath Biomarkers by Sensation Technology

Analysis of breath for various respiratory biomarkers is an emerging field for Sensation™ technology (Nanomix Inc), which is a nanoelectronic detection platform based on carbon nanotube networks. It consists of a tiny detector chip with one or more individually addressable detection elements, each capable of being independently functionalized to detect a specific target analyte. Sensation™ technology is enabling a new noninvasive device for asthma monitoring by measuring the level of nitric oxide (NO) in exhaled breath.

Detection of Breath Biomarkers by Nanosensors

To develop breath testing for identifying Alzheimer's disease (AD) and Parkinson's disease (PD), alveolar breath was collected from volunteers (AD patients, PD patients and healthy controls) and analyzed using combinations of nanomaterial-based sensors (Tisch et al. 2013). Discriminant factor analysis was applied to detect

statistically significant differences between study groups and classification success was estimated using cross-validation. The pattern identification was supported by chemical analysis of the breath samples using gas chromatography combined with MS. The combinations of sensors could clearly distinguish AD from healthy states, PD from healthy states, and AD from PD states, with a classification accuracy of 85, 78 and 84%, respectively. Gas chromatography combined with MS analysis showed statistically significant differences in the average abundance of several volatile organic compounds in the breath of AD, PD and healthy subjects, thus supporting the breath prints observed with the sensors. Conclusion is that breath prints identified with combinations of nanomaterial-based sensors have future potential as cost-effective, fast and reliable biomarkers for AD and PD.

Detection of Breath Biomarkers Optical Frequency Comb Spectroscopy

Broad-bandwidth, high-spectral-resolution optical detection of human breath has used to identify multiple important biomarkers correlated with specific diseases and metabolic processes. This optical-frequency-comb-based breath analysis system has shown excellent performance in high detection sensitivity, ability to identify and distinguish a large number of analytes, and simultaneous, real-time information processing. A series of breath measurements have been done with this method including stable isotope ratios of CO_2, breath concentrations of CO, and the presence of trace concentrations of NH_3 in high concentrations of H_2O. It will enable detection of molecules that may be biomarkers for diseases like asthma or cancer by blasting a person's breath with laser light. New designs of prism cavities promise to expand the spectral coverage of optical cavities from 200 nm to several microns, greatly increasing the number of biomarkers that can be measured by a single system. Even the currently available system can be used for clinical trials to gather statistics for the feasibility and cost effectiveness of breath measurements for non-invasive health screening tests.

Detection of Breath Biomarkers by Infrared Absorption Spectroscopy

Oxford Medical Diagnostics (OMD) is developing the Acetone Breath Analyzer, which is based on its infrared absorption spectroscopy technology based on CEAS (Cavity Enhanced Absorption Spectroscopy) technology. An optical cavity with ultra-high grade mirrors at both ends, enables repetitive wavelength scanning over the targeted region of absorption. It will be used to screen and diagnose type 1 and type 2 diabetes and monitor weight loss in healthy individuals. In persons with diabetes mellitus, lack of proper glucose metabolism leads to utilization of fat with resulting production of acetone, a biomarker of diabetes, which can be detected in

breath. The firm uses mass spec technology (V&F Analyse- und Messtechnik) as the gold standard with which to compare clinical results from diabetic patients with its laser-based breath products.

Detection of Biomarkers by Electronic Nose

An electronic nose (ENose) developed for air quality monitoring at the Jet Propulsion Laboratory (Pasadena, CA) has been used to distinguish between the odors of brain and brain tumor tissues (Kateb et al. 2009). The ENose was able to distinguish between the two types of organ tissue and between the two types of tumor cell lines. The variation in array response for the organ tissues was 19% and between the two types of cultured cell lines was 22%. Odor signatures of different cells as well as cellular proliferation, growth, differentiation and infiltration are being studied. The evaluation of exhaled breath profiles by ENose) is a promising non-invasive diagnostic tool, and the discrimination of breathprints between patients with COPD and asthma has been reported.

Fluorescent Indicators for Biomarkers

In earlier years scientists tagged samples – whether nucleic acid, protein, cell, or tissue – with radioactive labels, and captured images on film. Safety concerns, convenience, and sensitivity, spurred the development of alternative techniques, and today, researchers can choose from a range of options, including fluorescence. Fluorescence occurs when light is absorbed from an external (excitation) source by a fluorescent molecule (fluorophore) and subsequently emitted. The cycle of excitation and emission will continue until the excitation source is turned off or the fluorophore is consumed in a chemical reaction. A fluorescent probe is any small molecule that that undergoes changes in one or more of its fluorescent properties as a result of noncovalent interaction with a protein or other macromolecular structure. There are many different fluorescent molecules, both naturally occurring and synthetic, each molecule having distinctive spectroscopic properties. This variety of molecules can be exploited to enable highly localized, sensitive detection of chemical reactions, for tagging and identification of molecular structures, for monitoring of cellular states, and even for monitoring multiple properties simultaneously. Due to their higher sensitivity than chromogenic dyes, the fluorescent probes can be used to effectively signal the presence of minute amounts of a specific protein, DNA or RNA without the hazards associated with radioactive labels.

Simple water-soluble lanthanum and europium complexes are effective at detecting neutral sugars as well as glycolipids and phospholipids. In solutions at physiologically relevant pH the fluorescent lanthanum complex binds neutral sugars with apparent binding constants comparable to those of arylboronic acids.

Interference from commonly occurring anions is minimal. The europium complex detects sialic acid-containing gangliosides at pH 7.0 over an asialoganglioside. This selectivity is attributed, in large part, to the cooperative complexation of the oligosaccharide and sialic acid residues to the metal center, based on analogous prior studies. Lysophosphatidic acid (LPA), a biomarker for several pathological conditions including ovarian cancer, can be selectively detected by the europium complex. LPA is also detected via a fluorescence increase in human plasma samples. The 2-sn-OH moiety of LPA plays a key role in promoting binding to the metal center. Other molecules found in common brain ganglioside and phospholipid extracts do not interfere in the ganglioside or LPA fluorescence assays.

Molecular Imaging Technologies

Imaging has a long history in medicine. Several technologies are used for imaging in vivo. X-rays are the oldest form of in vivo imaging and form the basis of computer tomography imaging. The current focus in relation to imaging biomarkers is on molecular imaging, which is defined as the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in humans and other living systems. Molecular imaging typically includes 2D or 3D imaging as well as quantification over time. The techniques used include radiotracer imaging/nuclear medicine, MRI/MRS, optical imaging, ultrasound and others.

Computer Tomography

In computer tomography (CT) x-rays are emitted from a source and received by a set of detectors on the opposing side of the object. The source and detectors revolve around the patient continuously while the patient lies on a robotically controlled gantry. The resulting spiral CT scan is processed through interpolation and back-projection algorithms to form a series of cross-sectional images which, taken together, form a three-dimensional dataset representing the object of interest.

CT offers high resolution and fast acquisition of data, and is suited for studies that require precise characterizations of bony structures, such as the spine, or for cases in which image acquisition duration is of crucial importance, such as trauma. High-resolution CT is also preferred for characterizing lesions and other abnormalities within the chest and vascular structures, as well as many applications within the gastrointestinal and genitourinary regions. In the context of drug development, CT is a valuable tool for quantifying lesion size and location, particularly within the chest and abdominal regions. The image resolution of CT is >0.5 mm. More recently, by tracking the appearance of contrast-enhancing dyes as they wash into an organ, CT perfusion has gained greater applicability and utility.

Magnetic Resonance Imaging

In contrast to the use of ionizing radiation in CT, magnetic resonance imaging (MRI) uses radio-frequency pulses and magnetic fields to obtain signals from changes in nuclear magnetic moments. Specifically, as the alignment and relaxation of protons occur in response to pulsed radio-frequencies, characteristic relaxation times can be measured, most notably T1 (the longitudinal relaxation time) and T2 (the transverse relaxation time). Whereas CT images are characterized by a single parameter, namely the x-ray attenuation of the tissue along the propagation path, MRI is characterized by far more parameters, including proton density, T1, T2, flow, diffusion and susceptibility, among others. It is this flexibility that makes the use of MRI a double-edged sword. MRI is useful in numerous applications, but repeatability and standardization in multicenter clinical trials can be challenging. Although lower in resolution and requiring more time for data acquisition than that by CT, MRI offers superior soft tissue contrast, making MRI the modality of choice in the brain, in addition to specific applications in musculoskeletal and gastrointestinal systems. MRI, with and without contrast agents, is also used for numerous functional assessments, including tissue perfusion, diffusion, tumor permeability, and blood oxygenation level-dependent (BOLD) fMRI studies. A technique based on the same principle as MRI, but providing a greater degree of molecular characterization is magnetic resonance spectroscopy (MRS), where spectroscopic profiles of the chemical constituents within a sample are obtained. Real-time molecular imaging with MRI is possible now as demonstrated by imaging of the distribution of pyruvate and mapping of its major metabolites lactate and alanine within a time frame of ~10 s.

Positron Emission Tomography

Radionuclide imaging uses bi-functional agents containing a radiolabel that confers detectability, and a chemical and/or pharmaceutical moiety that determines uptake and distribution in the body. In the case of positron emission tomography (PET), the emitted positron passes through tissue and is ultimately annihilated when combined with an electron, resulting in two 511 keV photons emitted in opposite directions. Detectors are arranged in a ring around the tissue of interest, and only triggering events that arrive near-simultaneously at diametrically opposite detectors are recorded. Tomographic methods are then used to produce the resulting PET images.

Numerous radioisotopes are used for nuclear imaging. These tracer isotopes can be substituted into drug compounds directly to mimic naturally occurring compounds or can be complexed with other molecules to form new compounds referred to as radiopharmaceuticals. 2-18F-fluoro-2-deoxy-D-glucose (FDG), for example, is an analogue of glucose labeled with a positron-emitting form of fluorine and is used in PET imaging of metabolic activities that involve glucose uptake.

PET provides a noninvasive view into a person's living biology as it tracks a range of biological processes from metabolism to receptors, gene expression and drug activity. This imaging tool examines the chemistry and biology of a person's body by monitoring ingested tracer molecules, and it is used to study the metabolism of the brain, the heart and cancer.

Advantages of Imaging Biomarkers

Several characteristics of imaging biomarkers set them apart from other biomarkers. The advantages are:

- Imaging has been in routine use for diagnosis and disease management for several decades, and the ability to identify a wide spectrum of pathophysiology using imaging methods is well established.
- Imaging biomarkers tend to be much more closely associated with the expressed phenotype of diseases, thus enabling direct associations between therapy and effect.
- Functional imaging provides a dynamic picture of the disease.
- Imaging offers tremendous versatility for providing continuous, structural and functional assessments of therapy, offering snapshots of the bioactivity of drug compounds over time.
- Imaging provides therapy assessments in animals and humans alike, and is therefore an important tool for promoting translational research.
- Imaging has now entered molecular era with molecular imaging and nanoparticles as contrast media.

Monitoring In Vivo Gene Expression by Molecular Imaging

Molecular imaging is an emerging field of study that deals with imaging of disease on a cellular and molecular level. It can be considered as an extension of molecular diagnostics. In contradistinction to "classical" diagnostic imaging, it sets forth to probe the molecular abnormalities that are the basis of disease rather than to image the end effects of these molecular alterations. Radionuclide imaging, MRI, and PET can be used visualize gene expression. Work done at the Beckman Institute/ California Institute of Technology (Pasadena, CA) deals with 3D MRI image of gene expression based on intracellular messenger concentration.

Several current in vitro assays for protein and gene expression have been translated into the radiologic sciences. Endeavors are under way to image targets ranging from DNA to entire phenotypes in vivo. The merging fields of molecular biology, molecular medicine, and imaging modalities may provide the means to

screen active drugs in vivo, image molecular processes, and diagnose disease at a presymptomatic stage. Role of imaging in drug discovery and development is described in Chap. 4.

Challenges and Future of Molecular Imaging

Basic Research in Molecular Imaging

Research leads clinical practice, and one of the challenges to the medical and regulatory communities is to facilitate the introduction of new imaging techniques into patient management. As technology advances, scientists will further their ability to use different probes with the whole spectrum of molecular imaging modalities to identify new targets within cells and associated with cell membranes and receptors and to quantify treatment effects on the expression of these biomarkers. Traditional tracer-based nuclear medicine research will be expanded within the molecular imaging arena to include optical imaging and magnetic resonance spectroscopy. These new imaging technologies, particularly associated with new probe development, can provide new contrast to medical imaging. Optical imaging is currently limited by its spatial resolution and imaging depth, and thermoacoustic tomography or radio frequency–based photoacoustic tomography is being developed to meet this challenge.

Imaging Intracellular NADH as a Biomarker of Disease

Reduced nicotinamide adenine dinucleotide (NADH) is a major electron donor in the oxidative phosphorylation and glycolytic pathways in cells. NADH fuels a series of biochemical reactions that involve various enzymes to produce ATP, the major energy source in cells. In the event of disease or a metabolic disorder, these enzymes and their related reactions can become disabled, causing a buildup of unused NADH.

Intrinsic NADH fluorescence has been employed as a natural probe for a range of cellular processes that include apoptosis, cancer pathology, and enzyme kinetics. Two-photon fluorescence lifetime and polarization imaging of intrinsic NADH in breast cancer and normal cells has been reported for quantitative analysis of the concentration and conformation of this coenzyme (Yu and Heikal 2009). Using a newly developed noninvasive assay, the authors estimated the average NADH concentration in cancer cells to be approximately 1.8-fold higher than in normal breast cells. Excess amounts of intracellular NADH, a naturally fluorescent molecule found in all living cells, could serve as a natural biomarker for cancer. These quantitative studies demonstrate the potential of dynamics (rather than intensity) imaging for probing mitochondrial anomalies associated with neurodegenerative diseases, cancer, diabetes, and aging. This approach is also applicable to other metabolic and signaling pathways in living cells, without the need for cell destruction as in conventional biochemical assays.

Devices for Molecular Imaging

An evolution in imaging technology is occurring and will continue as imaging capabilities continue to expand from the anatomical to the functional and to the molecular. The expansion of imaging capabilities will enable the identification of imaging probes specific for molecular processes, and new multimodality imaging technologies will be developed to appropriately utilize these new probes, focusing on normal and abnormal biological processes. The future will bring nanoparticle delivery vehicles to deliver gene therapy to patients, smart contrast agents, target-specific optical agents and stem cell-based imaging therapy.

Imaging Biomarkers in Clinical Trials

SNM (http://www.snm.org/), an international scientific and medical association dedicated to advancing molecular imaging and therapy, created the Molecular Imaging Clinical Trials Network in response to the need for streamlined processes to utilize imaging biomarkers in clinical trials and clinical practice. There is widespread agreement that the use of imaging biomarkers in the drug development process can significantly reduce this burden and speed the timelines to clinical use. To specifically address this opportunity, SNM has designed a first-of-its-kind model for the use of imaging biomarkers in clinical trials that spans drug development, molecular imaging, radiolabeled probe development and manufacturing and regulatory issues to integrate the use of investigational imaging biomarkers into multicenter clinical trials.

Molecular Imaging in Clinical Practice

Work will continue to examine and validate future clinical applications for FDG PET/CT for oncology (diagnosis and staging, treatment planning and response, detection of recurrent or residual disease, restaging), for myocardial perfusion (coronary artery disease, myocardial viability), and for neurology and neurosurgery (brain tumors, medically intractable epilepsy, stroke, movement disorders, Alzheimer's disease and other dementias). Bioluminescence imaging, which enables visualization of genetic expression and physiological processes at the molecular level in living tissues, can identify specific gene expression in cancer cells and may be used to identify metastatic potential.

Nuclear Magnetic Resonance

High-resolution nuclear magnetic resonance (NMR) spectroscopy is a quantitative technique that can report on hundreds of compounds in a single measurement. The ubiquitous presence of hydrogen in biomolecules as well as a favorable isotope

distribution and magnetic sensitivity make 1H NMR the obvious choice for generating profiles; however, there are NMR-visible isotopes for most chemical elements, including 13C, 31P, and 15N. Modern cryogenically cooled probes and capillary probes can push limits of detection by NMR to nanomolar levels or samples volumes as low as 1.5 mL. This enables metabonomics to be applied to a number of volume or mass limited circumstances, such as the use of microdialysates or regular blood sampling from small animals without sacrificing them.

Sample preparation for routine biofluid analysis is minimal. The use of magic angle spinning NMR (MAS-NMR) enables intact tissues and cells to be examined with little or no preparation and on as little as 20 mg of material. Profiles generated via MAS-NMR can also reveal the effects of toxin treatment on the metabolite composition within different cellular environments either via diffusion and relaxation measurements in intact tissue or by comparing NMR profiles generated by various tissue isolates. The ability to localize biochemical changes to a specific tissue, cell type, or organelle provides valuable information pertaining to the mechanism of toxicity and can aid the interpretation of biofluid analyses. MAS-NMR may provide a means to validate in vitro systems in terms of in vivo metabolic responses to toxicity.

Chemical Derivatization to Enhance Biomarker Detection by NMR

A simple chemical reaction has been developed Purdue University (Lafayette, IN) to improve the ability to detect important molecules in complex fluids such as blood, rendering the biomarkers for some genetically caused metabolic disorders up to 100 times more visible. The chemical derivatization method selects a class of metabolites from a complex mixture and enhances their detection by ^{13}C NMR. Acetylation of amines directly in aqueous medium with 1,1'-(13)C(2) acetic anhydride is a simple method that creates a high sensitivity and quantitative label in complex biofluids with minimal sample pretreatment. Detection using either 1D or 2D ^{13}C NMR experiments produces highly resolved spectra with improved sensitivity. Experiments to identify and compare amino acids and related metabolites in normal human urine and serum samples as well as in urine from patients with the inborn errors of metabolism tyrosinemia type II, argininosuccinic aciduria, homocystinuria, and phenylketonuria have demonstrated the usefulness of this method. The use of metabolite derivatization and ^{13}C NMR spectroscopy produces data suitable for metabolite profiling analysis of biofluids on a time scale that allows routine use. Extension of this approach to enhance the NMR detection of other classes of metabolites has also been accomplished. The improved detection of low-concentration metabolites creates opportunities to improve the understanding of the biological processes and develop improved disease detection methods.

Fluxomics by Using NMR

Fluxomics is measurement of flux rates of biomarkers using NMR with stable isotope precursors. NMR can do non-invasive monitoring of live tissue and repetitive sampling (longitudinal) without sacrifining animals. The technology can be translated directly to humans. NMR and ^{1}H spectroscopic imaging (MRSI) can make in vivo fluxomic measurements whereas LC-MS can be used to identify less abundant metabolites. This approach has been used for human prostate to differentiate between cancer and benign hypertrophy. Advantages of this method are:

- Fluxomics is more sensitive than concentration alone.
- Fluxomics completes the system biology equation permitting mechanistic modeling and anchoring proteomic and transcriptomic data.

Nanobiotechnology

Nanotechnology (Greek word nano means dwarf) is the creation and utilization of materials, devices, and systems through the control of matter on the nanometer-length scale, i.e., at the level of atoms, molecules, and supramolecular structures. It is the popular term for the construction and utilization of functional structures with at least one characteristic dimension measured in nanometers – a nanometer is one billionth of a meter (10^{-9} m). This is roughly four times the diameter of an individual atom and the bond between two individual atoms is 0.15 nm long. Proteins are 1–20 nm in size. The definition of 'small', another term used in relation to nanotechnology, depends on the application, but can range from 1 to 1 mm). Nano is not the smallest scale; further down the power of ten are angstrom (=0.1 nm), pico, femto, atto and zepto. By weight, the mass of a small virus is about 10 attograms. An attogram is one-thousandth of a femtogram, which is one-thousandth of a picogram, which is one-thousandth of a nanogram. Nanobiotechnologies are described in a special report on this topic (Jain 2017c). The Handbook of Nanomedicine describes applications of nanobiotechnology for healthcare (Jain 2017a).

Dip Pen Nanolithography

Dip pen nanolithography (DPN), originally developed by the Mirkin Lab at Northwestern University in USA, uses the tip of an AFM to write molecular "inks" directly on a surface. Biomolecules such as proteins and viruses can be positioned on surfaces to form nanoarrays that retain their biological activity.

Multiple-allergen testing for high throughput and high sensitivity requires the development of miniaturized immunoassays that can be performed with minute amounts of test analyte that are usually available. Construction of such miniaturized

biochips containing arrays of test allergens needs application of a technique that can deposit molecules at high resolution and speed while preserving their functionality. DPN is an ideal technique to create such biologically active surfaces, and it has already been successfully applied for the direct, nanoscale deposition of functional proteins, as well as for the fabrication of biochemical templates for selective adsorption. It has potential applications for detection of allergen-specific immunoglobin E (IgE) antibodies and for mast cell activation profiling.

High-throughput protein arrays allow the miniaturized and parallel analysis of large numbers of diagnostic biomarkers in complex samples. This capability can be enhanced by nanotechnology. DPN technique has been extended to protein arrays with features as small as 45 nm and immunoproteins as well as enzymes can be deposited. Selective binding of antibodies to protein nanoarrays can be detected without the use of labels by monitoring small (5–15 nm) topographical height increases in AFM images.

Advantages of this system include:

- Ultrasensitive detection attains single femtograms/ml sensitivity.
- Generate more data with less sample: low-abundance biomarker studies on rare and hard-to-collect samples are possible.
- Enables multiplex, high-throughput protein analysis: an entire slide with up to 96 sub-arrays can be scanned in 3.5 min
- Reduces protein assay costs: femtofluidic sample and reagent volumes deliver rapid reaction kinetics and minimize expenditure.

Nanomaterials for Biolabeling

Nanomaterials are suitable for biolabeling. Nanoparticles usually form the core in nanobiomaterials. However, in order to interact with biological target, a biological or molecular coating or layer acting as an interface needs to be attached to the nanoparticle. Coatings that make the nanoparticles biocompatible include antibodies, biopolymers or monolayers of small molecules. A nanobiomaterial may be in the form of nanovesicle surrounded by a membrane or a layer. The shape is more often spherical but cylindrical, plate-like and other shapes are possible. The size and size distribution might be important in some cases, for example if penetration through a pore structure of a cellular membrane is required. The size is critical when quantum-sized effects are used to control material properties. A tight control of the average particle size and a narrow distribution of sizes allow creating very efficient fluorescent probes that emit narrow light in a very wide range of wavelengths. This helps with creating biomarkers with many and well distinguished colors. The core itself might have several layers and be multifunctional. For example, combining magnetic and luminescent layers one can both detect and manipulate the particles.

The core particle is often protected by several monolayers of inert material, for example silica. Organic molecules that are adsorbed or chemisorbed on the surface

of the particle are also used for this purpose. The same layer might act as a biocompatible material. However, more often an additional layer of linker molecules is required that has reactive groups at both ends. One group is aimed at attaching the linker to the nanoparticle surface and the other is used to bind various biocompatible substances such as antibodies depending on the function required by the application. Water-soluble, biocompatible, fluorescent, and stable silver/dendrimer nanocomposites have been synthesized that exhibit a potential for labeling cells in vitro as cell biomarkers.

Efforts to improve the performance of immunoassays and immunosensors by incorporating different kinds of nanostructures have gained considerable momentum over the last decade. Most of the studies focus on artificial, particulate marker systems, both organic and inorganic. Inorganic nanoparticle labels based on noble metals, semiconductor quantum dots and nanoshells appear to be the most versatile systems for these bioanalytical applications of nanophotonics. The underlying detection procedures are more commonly based on optical techniques. These include nanoparticle applications generating signals as diverse as static and time-resolved luminescence, one- and two-photon absorption, Raman and Rayleigh scattering as well as surface plasmon resonance and others. In general, all efforts are aimed to achieve one or more of the following goals:

- Lowering of detection limits (if possible, down to single-molecule level)
- Parallel integration of multiple signals (multiplexing)
- Signal amplification by several orders of magnitude
- Prevention of photobleaching effects with concomitant maintenance of antigen binding specificity and sensitivity.

Quantum dot Molecular Labels

Quantum dots (QDs) are crystalline semiconductors composed of a few hundred or thousand atoms that emit different colors of light when illuminated by a laser. Stable QDs are made from cadmium selenide and zinc sulfide. Because these probes are stable, they have the ability to remain in a cell's cytoplasm and nucleus without fading out much longer than conventional fluorescent labels. This could give biologists a clear view of processes that span several hours or even days, such as DNA replication, genomic alterations, and cell cycle control. Their longevity has also made QDs a molecular label, allowing scientists to study the earliest signs of cancer, track the effectiveness of pharmaceuticals that target the cellular underpinnings of disease, and understand the events that occur during stem cell differentiation.

One drawback to this approach, however, is that these QDs may release potentially toxic cadmium and zinc ions into cells. To solve this problem, QDs are coated with a protective layer of polyethylene glycol (PEG), which is a very nonreactive and stable compound that is used extensively by the pharmaceutical industry in drug formulation. This layer is designed to prevent the dots from leaking heavy metal ions into cells once they are inside. The tool used test the safety of QDs is a gene

chip packed with 18,400 probes of known human genes and it is a comprehensive method to measure the toxicity of nanoscale particles. This chip is designed to enable the researchers to expose the human genome QDs, and determine the extent to which the compound forces the genes to express themselves abnormally.

Specially coated QDs fluorescent nanoprobes affect only a tiny fraction of the human genome, dispelling the concern that the mere presence of these potentially toxic sentinels disrupts a cell's function. Moreover, the affected genes are not related to heavy metal exposure, which would be the case if the cells had been exposed to cadmium or zinc ions. Because of their protective coating, QDs have minimal impact on cells; the only gene changes are in transporter proteins, which is expected because the dots have to be transported into and within the cell.

PEG-coated QDs are used for in vivo imaging of breast and prostate cancer. These cancers could be detected at very early stages and the molecular makeup can be characterized for effective treatment.

Bioconjugated QDs for Multiplexed Profiling of Biomarkers

Bioconjugated QDs, collections of different sized nanoparticles embedded in tiny polymer beads, provide a new class of biological labels for evaluating biomarkers on intact cells and tissue specimens. In particular, the use of multicolor QD probes in immunohistochemistry is considered one of the most important and clinically relevant applications. Bioconjugated QDs can be used for multiplexed profiling of biomarkers, and ultimately for correlation with disease progression and response to therapy. This will increase the clinician's ability to predict the likely outcomes of drug therapy in a personalized approach to disease management. Bioinformatics and systems biology is used to link each individual's molecule profile with disease diagnosis and treatment decisions. The usefulness of these protocols was demonstrated by simultaneously identifying multiple biomarkers in prostate cancer tissue. In general, QD bioconjugation is completed within one day, and multiplexed molecular profiling takes 1–3 days depending on the number of biomarkers and QD probes used.

Magnetic Nanotags for Multipley Detection of Biomarkers

Magnetic nanotags (MNTs) are a promising alternative to fluorescent labels in biomolecular detection assays, because minute quantities of MNTs can be detected with inexpensive sensors. Probe sensors are functionalized with capture antibodies specific to the chosen analyte. During analyte incubation, the probe sensors capture a fraction of the analyte molecules. A biotinylated linker antibody is subsequently incubated and binds to the captured analyte, providing binding sites for the streptavidin-coated MNTs, which are incubated further. The nanotag binding signal, which saturates at an analyte concentration-dependent level, is used to quantify the analyte concentration. However, translation of this technique into easy-to-use and multilplexed protein assays, which are highly sought after in molecular

diagnostics such as cancer diagnosis and personalized medicine, has been challenging. Multiplex protein detection of potential cancer biomarkers has been demonstrated at subpicomolar concentration. With the addition of nanotag amplification, the analytic sensitivity extends into the low femtomolar concentration range. The multianalyte ability, sensitivity, scalability, and ease of use of the MNT-based protein assay technology make it a strong contender for versatile and portable molecular diagnostics in both research and clinical settings.

Nanoparticles for Molecular Imaging

Although developments in molecular imaging have been dominated by nuclear medicine agents in the past, the advent of nanotechnology led to MRI molecular agents that enable detection of sparse biomarkers with a high-resolution imaging. A wide variety of nanoparticulate MRI contrast agents are available, most of which are superparamagnetic iron oxide-based constructs. Perfluorocarbon (PFC) nanoparticulate platform is not only effective as a T1-weighted agent, but also supports ^{19}F magnetic resonance spectroscopy and imaging. The unique capability of ^{19}F permits confirmation and segmentation of MRI images as well as direct quantification of nanoparticle concentrations within a voxel.

Ultrasmall superparamagnetic iron oxide (USPIO) is a cell-specific contrast agent for MRI. An open-label phase II study has tested the potential of USPIO-enhanced MRI for macrophage imaging in human cerebral ischemic lesions. USPIO-induced signal alterations throughout differed from signatures of conventional gadolinium-enhanced MRI, thus being independent from breakdown of the blood-brain barrier. Macrophages, as the prevailing inflammatory cell population in stroke, contribute to brain damage. USPIO-enhanced MRI may provide an in vivo surrogate marker of cellular inflammation in stroke and other CNS pathologies. USPIO has favorable properties that result from its intravascular retention and lack of extravasation, allowing optimal contrast between the vessel and the adjacent tissue for several minutes postinjection. SH U 555 C (Bayer AG) is an optimized formulation of carboxydextran-coated ferucarbotran (Resovist) is another USPIO. When injected intravenously, it can depict first-pass MR angiography and cardiac perfusion.

Nanoparticles for Discovering Biomarkers

Most of the applications of nanoparticles have focused on imaging systems and drug delivery vectors. The physicochemical characteristics and high surface areas of nanoparticles also make them ideal candidates for developing biomarker harvesting platforms. Given the variety of nanoparticle technologies that are available, it is feasible to tailor nanoparticle surfaces to selectively bind a subset of biomarkers and

sequestering them for later study using high sensitivity proteomic tests. Biomarker harvesting is an underutilized application of nanoparticle technology and is likely undergo substantial growth.

Functional polymer-coated nanoparticles can be used for quick detection of biomarkers and DNA separation. Multiarray of hepatocyte spheroids on a microfabricated polymer-brush surface can maintain the hepatocyte viability and liver-specific functions, offering a new high-throughput screening method of pharmacologically and toxicologically active compounds for drug discovery.

Nanoparticles are being used for the electrochemical detection of proteins taking advantage of their versatility and inherent electrochemistry as modifiers of the electrotransducer surfaces. The improved electrochemical response is either due to enhancement of the electron transference or the increased efficiency of immobilization of biomolecules. Although nanoparticle-based electrochemical immunosensors have shown high sensitivity and selectivity their application in clinical analysis still needs rigorous testing to evaluate these advantages in comparison to classical assays in terms of reproducibility, stability and cost while being applied for real sample analysis (de la Escosura-Muñiz and Merkoçi 2010).

Nanoproteomics and Biomarkers

Nanoproteomics – application of nanobiotechnology to proteomics – improves on most current protocols including protein purification/display and automated identification schemes that yield unacceptably low recoveries with reduced sensitivity and speed while requiring more starting material. Low abundant proteins and proteins that can only be isolated from limited source material (e.g. biopsies) can be subjected to nanoscale protein analysis – nano-capture of specific proteins and complexes, and optimization of all subsequent sample handling steps leading to mass analysis of peptide fragments. This is a focused approach, also termed targeted proteomics, and involves examination of subsets of the proteome, e.g., those proteins that are either specifically modified, or bind to a particular DNA sequence, or exist as members of higher order complexes, or any combination thereof. This approach is used at Memorial Sloan-Kettering Cancer Center and Cornell University, New York, to identify how genetic determinants of cancer alter cellular physiology and response to agonists.

High-Field Asymmetric Waveform ion Mobility Mass Spectrometry

An ion mobility technology – high-field asymmetric waveform ion mobility mass spectrometry (FAIMS) – has been introduced as online ion selection methods compatible with electrospray ionization (ESI). FAIMS uses ion separation to improve detection limits of peptide ions when used in conjunction with electrospray and nanoelectrospray MS. This facilitates the identification of low-abundance peptide

ions often present at ppm levels in complex proteolytic digests and expand the sensitivity and selectivity of nanoLC–MS analyses in global and targeted proteomics approaches. This functionality likely will play an important role in drug discovery and biomarker programs for monitoring of disease progression and drug efficacy.

Nanosensors for Measuring Biomarkers in Blood

To overcome the challenge of detection of biomarkers in whole blood, Yale University researchers have developed a lable-free nanosensor. A microfluidic purification chip simultaneously captures multiple biomarkers from blood samples and releases them, after washing, into purified buffer for sensing by a silicon nanoribbon detector. This two-stage approach isolates the detector from the complex environment of whole blood, and reduces its minimum required sensitivity by effectively pre-concentrating the biomarkers. Deposit of antigens on the chip enables detection down to extremely small concentrations. Specific and quantitative detection of two model cancer antigens – antigens specific to prostate and breast cancer – from a 10 μl sample of whole blood was carried out in less than 20 min on the order of picograms per milliliter, with 10% accuracy (Stern et al. 2010). The device may be used at POC to test for a wide range of disease biomarkers at the same time, from ovarian cancer to cardiovascular disease. This low-cost technology can be rapidly translated into clinical application.

Nanobiochip Sensor Technique for Analysis of Oral Cancer Biomarkers

A pilot study has described a nanobiochip sensor technique for analysis of oral cancer biomarkers in exfoliative cytology specimens, targeting both biochemical and morphologic changes associated with early oral tumorigenesis (Weigum et al. 2010). Oral lesions from dental patients, along with normal epithelium from healthy volunteers, were sampled using a noninvasive brush biopsy technique. Specimens were enriched, immunolabeled, and imaged in the nanobiochip sensor according to previously established assays for the epidermal growth factor receptor (EGFR) biomarker and cytomorphometry. Four key parameters were significantly elevated in both dysplastic and malignant lesions relative to healthy oral epithelium, including the nuclear area and diameter, the nuclear-to-cytoplasmic ratio, and EGFR biomarker expression. Further examination using logistic regression and receiver operating characteristic curve analyses identified morphologic features as the best predictors of disease individually, whereas a combination of all features further enhanced discrimination of oral cancer and precancerous conditions with high sensitivity and specificity. Further clinical trials are necessary to validate the regression model and

evaluate other potential biomarkers. Nanobiochip sensor technique is a promising tool for early detection of oral cancer, which could enhance patient survival.

Future Prospects of Application of Nanobiotechnology for Biomarkers

Nanobiotechnology is progressing rapidly and the impact will be felt on discovery of biomarkers. Nanotechnology offers the possibility to create devices which can screen for disease biomarkers at very fast rates. The tools will be developed by identifying biomarkers for particular diseases that can then lead to diagnostic tests. One project in this area draws together the expertise of a team of researchers from the Australian Institute for Bioengineering and Nanotechnology at The University of Queensland (UQ), the Fred Hutchinson Cancer Research Centere (Seattle, WA), and the Seattle Biomedical Research Institute. This project is supported by a contribution of $2 million from the Queensland State Government through the National and International Research Alliances Program. In addition to Alliances funding, the project will receive support from the participating institutes and UQ spin-off company Nanomics Biosystems Pty Ltd.

Scientists at the California Institute of Technology (Pasadena, CA) are pursuing an approach to early detection of cancer that is based on tiny circuits with nanosize transistors. Each transistor can be attached to an antibody, a biological molecule specially designed to attach to a biomarker. When the antibody binds to the biomarker, the transistor's ability to conduct electricity changes slightly, signaling the biomarker's presence. The long-term goal is to build a circuit analogous to a microscopic computer chip that can detect hundreds or thousands of biomarkers in a single test that could catch early cancers that would otherwise go undetected.

Bioinformatics

The deluge of genomic and proteomic data in the past two decades has driven the development of bioinformatics as well as creation of tools that search and analyze biomolecular sequences and structures. Bioinformatics is highly interdisciplinary, using knowledge from mathematics, statistics, computer science, biology, medicine, physics, chemistry, and engineering. Scientists at the Medical University of South Carolina (Charleston, SC) have filed provisional patent application for a method that utilizes machine learning techniques to select biomarkers that show discriminating power and applies statistical, mathematical, or computational tools for the derivation of the patients' information. The aim is identification of biomarkers that can predict progression of a disease and would aid in targeting aggressive therapy to those patients that could benefit the most from treatment that would slow or stop progression of a disease. Some of the other approaches are described here.

Biomarker Workflow Guide

Biomarker Workflow Guide (Ingenuity Systems Inc) is used to identify potentially useful biomarkers during the preclinical exploratory phase via analysis of gene expression array data or proteomics data. With the workflow guide, researchers can more easily prioritize biomarker leads by associating them with biology relevant to the observed phenotype. IPA can also help researchers select biomarker panels from the most significant clusters by grouping clusters in the context of pathways, functions or diseases. Computational pathways analytics was used to develop network signatures from gene expression data and to identify osteopontin as a biomarker candidate for mesothelioma. It is expected to become a standard protocol for biomarker discovery.

Analysis of Microarray Data for Selecting Useful Biomarkers

DNA microarrays are used for studying gene expression. One potential application of this technology is the discovery of biomarkers for more accurate diagnosis and prognosis, and potentially for the earlier detection of disease or the monitoring of treatment effectiveness. Because microarray experiments generate a tremendous amount of data and because the number of laboratories generating microarray data is rapidly growing, new bioinformatics strategies that promote the maximum utilization of such data are necessary. Bioinformatic is used in the pattern analysis of serum biomarkers of breast and ovarian cancers.

Biomarker genes of human skin-derived can be identified by bioinformatic methods and DermArray DNA microarray analysis utilizing in vitro cultures of normal human epidermal keratinocytes, melanocytes, and dermal fibroblasts. Signature biomarker genes (up-regulated in one cell type) and anti-signature biomarker genes (down-regulated in one cell type) can be determined for the three major skin cell types. Many of the signature genes are known biomarkers for these cell types. Quantitative RT-PCR is used to verify signature biomarker genes. Several biomarkers of normal human skin cells have been identified, many of which may be valuable in diagnostic applications and as molecular targets for drug discovery and therapeutic intervention.

Role of Bioinformatics in Discovery of Protein Biomarkers

Developments in proteomic technology offer tremendous potential to yield novel biomarkers that are translatable to routine clinical use but major hurdles remain for translation into clinical application. There is a need for rigorous experimental design and methods to validate some of the unproven methods used currently. There is an

ongoing debate on where the burden of proof lies: statistically, biologically or clinically. There is no consensus about what constitutes a meaningful benchmark. It has been pointed out that statistical and machine learning methods are not a crutch for poor experimental design nor can they elucidate fundamental insight from poorly designed experiments. It is now clear that SELDI-TOF MS instrumentation used in the earlier proteomic pattern studies had insufficient resolution to enable the unambiguous identification of the putative biomarker molecules, which is needed if they are to be validated for forming the basis of a simplified, more widely adopted diagnostic. There is a need for calibration style benchmarking where the linearity of instrument responsiveness is established, to the ultimate benchmark – real clinical usage – as well as for many challenges in between, such as data normalization, peak detection, identification and quantification and, at some point, classification.

For non-hierarchically organized data in proteome databases, it is difficult to view relationships among biological facts. Scientists at Eli Lilly & co have demonstrated a platform where such data can be visualized through the application of a customized hierarchy incorporating medical subject headings (MeSH) classifications. This platform gives users flexibility in updating and manipulation. It can also facilitate fresh scientific insight by highlighting biological impacts across different hierarchical branches. They have integrated biomarker information from the curated Proteome database using MeSH and the StarTree visualization tool.

A novel framework has been presented for the identification of disease-specific protein biomarkers through the integration of biofluid proteomes and inter-disease genomic relationships using a network paradigm (Dudley and Butte 2009). This led to the creation of a blood plasma biomarker network by linking expression-based genomic profiles from 136 diseases to 1028 detectable blood plasma proteins. The authors also created a urine biomarker network by linking genomic profiles from 127 diseases to 577 proteins detectable in urine. Through analysis of these molecular biomarker networks, they found that the majority (>80%) of putative protein biomarkers are linked to multiple disease conditions and prospective disease-specific protein biomarkers are found in only a small subset of the biofluid proteomes. These findings illustrate the importance of considering shared molecular pathology across diseases when evaluating biomarker specificity. The proposed framework is amenable to integration with complimentary network models of biology, which could further constrain the biomarker candidate space, and establish a role for the understanding of multiscale, interdisease genomic relationships in biomarker discovery.

Role of Bioinformatics in Detection of Cancer Biomarkers

Cancer biomarkers are described in Chap. 13. Bioinformatics is applied for the exploration of cancer-related biomarkers, such as predisposition markers, diagnostics markers, prognostics markers, and therapeutics markers. Because quite large amounts of data are produced by the whole genome SNP scanning, bioinformatics

is necessary for the identification of SNPs associated with cancer predisposition. Individual cancer risks can be estimated accurately by detecting multiple SNPs affecting critical cancer-associated genes. Because expression profiles are determined by the signaling networks in cancer, network analyses promote the exploration of diagnostic biomarkers. Network analysis software using gene set enrichment program are developed by a variety of companies or groups; however, network analyses driven by human intelligence of experts is still powerful and more accurate. In the postgenomic era, bioinformatics is utilized for the identification of novel prognostic markers, recurrence prediction markers, and metastasis markers out of large amounts of genomics, transcriptomics, and proteomics data. Bioinformatics is utilized for the exploration of cancer-related biomarkers to select therapeutic optional choice among surgical operation, radiation therapy, and chemotherapy. Biomarkers for localized tumors with low metastatic potential support the selection of a surgical procedure, while biomarkers for infiltrating tumors with high metastatic potential support the select ion of radiation therapy and/or chemotherapy. Therapeutic biomarkers for squamous carcinoma of esophagus, lung, and cervical uterus have been characterized by using bioinformatics on transcriptomics data.

Biomarker Databases

References are given to several genomic, proteomic and metabolomic databases in this report and these are relevant to biomarkers. The value of such databases is recognized. Building of reliable biomarker databases and integration of information from the genome programs expands the scientific frontiers on etiology, health risk prediction and prevention of environmental disease. Biomarker validation may be performed in a number of ways: bench-side in traditional labs; web-based electronic resources such as gene ontology; literature databases; and clinical trial. Biomarker databases have potential value for pharmaceutical research and development.

The biomarker database GOBIOM (https://gobiomdb.com/gobiom/), contains >115,000 biomarkers (as of April 2017), including genomic, biochemical, imaging, metabolite, cellular, and other biomarkers, as well as data points from experimental, analytical, clinical, and statistical data with their qualifications under different medical interventions. The database includes information from >200,000 sources including clinical trials, scientific conferences, regulatory approval documents, literature databases, patents, etc. It covers 18 therapeutic areas and 1700 indications. It may be used in biomarker design and validation research. Under an agreement, the FDA uses GOBIOM as part of its Voluntary Exploratory Data Submission Program and in internal research projects. The Prevention of Organ Failure Center (PROOF Center) at the University of British Columbia, Canada, has agreed to use the GOBIOM to assess the commercialization potential of the its studies of biomarker development for heart, lung, and kidney failure.

Gene Networks as Biomarkers

Biomarkers are always parts of critical regulatory circuits either as players or as endpoint readouts. These roles as well as biological flexibility are generally accounted for by looking at groups of biomarkers that are linked to the situation observed but different subsets of biomarkers are found, which make it difficult to decide whether these results indicate the same basic situation. An innovative approach taken by Genomatix Software (Munich, Germany) elucidates the regulatory networks associated with a particular situation and then projects all the observed changes onto this network. This enables determination whether the two overlapping but distinct sets of biomarkers found are actually part of the same network. The gene network is thus a robust biomarker. Individual pathways in cancer are like lines that run through this network, which represents more stable manifestations than individual pathways. This approach has been applied to comparison of microarray date from freshly frozen and FFPE samples of prostate cancer from the same patient. Although these datasets look different, both belong to the same network. Another advantage of this approach is that analysis of pertinent data may enable detection of changes in the precancerous phase.

Role of Bioinformatics in Integrating Various Data and Biomarker Discovery

There is a need for integration of high-throughput biological data with less structured clinical data for discovery of biomarkers. A data integration strategy has been described that implements a clinical and biological database and a wiki interface (Sorani et al. 2010). The authors integrated parameters across clinical trials and associated genetic, gene expression and protein data to explore disease heterogeneity and develop predictive biomarkers. They undertook extensive clinical data standardization and biological data summarization to support biomarker discovery in rheumatoid arthritis. According to the authors of this study, a key enabler for future integration efforts will be the prospective adoption of standard clinical trial data nomenclature using a controlled vocabulary and ontology. Such standardization could facilitate future data loading, integration and cross-trial analysis and, ultimately, biomarker and drug discovery efforts.

Ariana Pharma's KEM® (knowledge extraction and management) rules-based data mining analytics software can be applied to identify predictive biomarkers of clinical adverse events, safety and efficacy. It can also identify biomarkers for potential applications in prognostic and companion diagnostic assays.

Evaluation of Biomarker Studies

Thousands of putative biomarkers have been discovered and there is a tremendous increase of publications on biomarkers in recent years. Many biomarkers are proposed in highly cited studies as determinants of disease risk, prognosis, or response

to treatment, but few are eventually transformed into clinical practice. A study has examined whether the magnitude of the effect sizes of biomarkers proposed in highly cited studies is accurate or overestimated (Ioannidis and Panagiotou 2011). In more than 80% of cases, the biomarker effect sizes were larger in the frequently cited studies than in the meta-analyses, with several of the associations failing to reach statistical significance in the largest studies available. The authors concluded that highly cited biomarker studies often report larger effect estimates for postulated associations than are reported in subsequent meta-analyses evaluating the same associations. The pattern was consistent suggesting that this is not an isolated occurrence for a single biomarker but a phenomenon that applies to many different biomarkers and relevant diseases in different settings.

Although the study design has limitations and does not cover all biomarkers, the findings could help explain why many biomarkers that appear to be promising do not enter the clinic. The authors of the study did not dispute the fact that some of the biomarkers covered in the study are important, but rigorous study design and analysis standards, coupled with ongoing analyses of biomarkers, are needed to verify biomarker associations and to accurately chronicle effect sizes. An editorial commenting on the study has pointed out that the findings should not discourage biomarker investigators, but advised caution about over-selling results from individual studies (Bossuyt 2011).

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Chapter 3
Biomarkers and Molecular Diagnostics

Introduction

Clinical application of molecular technologies for analysis of biomolecules to elucidate, diagnose and monitor human diseases is referred to as molecular diagnosis. It is a broad term and includes technologies that use DNA, RNA, genes or proteins as bases for diagnostic tests. Use of monoclonal antibodies (MAbs) and enzyme-linked immunosorbent assay (ELISA) is included in a broader term "in vitro diagnostics (IVD)", which also covers many applications not necessarily related to healthcare. "In vivo diagnostics" is the application of molecular diagnostics in the living subject, human or animal. It includes molecular imaging, which is the exploitation of specific molecules for image contrast and refers to the in vivo measurement and characterization of cellular and molecular level processes in animal or human subjects. There are over 600 molecular diagnostic systems of various types and this topic is covered in detail in a special report (Jain 2017). Molecular diagnostics overlaps with biomarkers. Biomarkers can be discovered with molecular diagnostic technologies and may also form the basis of molecular diagnostic tests.

Molecular Diagnostic Technologies

The most widely used molecular diagnostics technology has been the polymerase chain reaction (PCR) but several new technologies and non-PCR methods have been developed that are relevant to biomarkers.

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Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a method of nucleic acid analysis for producing large amounts of a specific DNA fragment of a defined sequence and length from a small amount of a complex template. It can selectively amplify a single molecule of DNA or RNA several millionfold in a few hours. Use of this technology enables the detection and analysis of specific gene sequences in a patient's sample without cloning. Analyses can be performed on even a few cells from body fluids or in a drop of blood. Thus, PCR eliminates the need to prepare large amounts of DNA from tissue samples. PCR has revolutionized molecular diagnostics. Apart from laboratory diagnosis, it has affected genomics and biotechnology as well. Because PCR is so well known, only basics of the technology are described here briefly.

Amplification

PCR is based on the enzymatic amplification of a fragment of DNA that is flanked by two "primers"--short oligonucleotides that hybridize to the opposite strands of the target sequence and then prime synthesis of the complementary DNA sequence by DNA polymerase (an enzyme). The chain reaction is a three-step process--denaturation, annealing, and extension that is repeated in several cycles. At each stage of the process, the number of copies is doubled--from two, to four, to eight, and so on. The reactions are controlled by changing the temperature using a special heat-stable Taq polymerase. After 20 cycles, roughly 1 million copies exist, or enough material to detect the desired DNA by conventional means such as color reaction.

RNA can also be studied by making a DNA copy of the RNA using the enzyme reverse transcriptase. Such an approach enables the study of mRNA in cells that use the molecule to synthesize specific proteins or the detection of the genome of RNA viruses. PCR has been fully automated via use of thermal cycling. It is a fast, sensitive, and specific test with applications in diagnosis of various diseases. Some innovations of PCR are described in the following sections.

Target Selection

Several strategies are available for selecting a genetic target to be amplified so as to detect an infectious disease organism. For example, genes that contain both conserved and variable sequence regions may be targeted. In such a case, specificity may be obtained either at the amplification (primer) or detection (probe) stage. The target may also consist of a virulent gene that is uniquely responsible for distinguishing pathogenic from closely related nonpathogenic strains, types, or species.

Detection of Amplified DNA

The first detection methods used with PCR were radioactively labeled probes that identified specific amplified sequences. With improvements in specificity, it became possible to visualize amplified DNA of the predicted size directly by examining its fluorescence after staining. Probes have now been converted to nonisotopic colorimetric systems. In another approach, the probe is a "reverse" component (bound to a membrane) and "captures" a specific allele or a sequence variant if it is present in the amplified DNA.

An alternative to probe-based detection system relies on labeled primers and strives for perfect target specificity in the amplification reaction. This process is straightforward if the target gene differs from the unintended targets by a deletion or gene rearrangement. Using the Duchenne muscular dystrophy gene deletions as a model, researchers have now automated this method. It should prove highly useful in forensic investigations for rapid analysis of amplified targets that differ in length, such as variable number tandem repeat (VNTR) loci.

In general, nonradioactive detection systems fall into two classes – direct and indirect – based on the detectability of the label. In most indirect detection methods, the primary label (e.g., biotin) is identified through its interaction with a secondary system that contains a detectable reporter group. Various techniques for direct detection of nucleic acids include the following:

- Direct enzymatic detection, which requires the construction of enzyme DNA conjugates.
- Fluorescent detection, which depends on the ability to synthesize fluorescent DNA. This technique may emerge as the detection technology of choice in future PCR systems.
- Chemiluminescent detection via direct attachment of chemiluminescent labels (e.g., acridium esters and isoluminol derivatives) to synthetic nucleotides.

Limitations of PCR

The DNA polymerases bind to short double-stranded DNA fragments without sequence specificity. The presence of endogenous amplicon DNA accumulated in later PCR cycles completely inhibits the activity of DNA polymerase. PCR has reached a sort of plateau phase and the main factor contributing to this is binding of DNA polymerase to its amplification products.

While there have been attempts to use internal amplification standards and hybridization with sequence-specific oligonucleotides to improve PCR quantification, the difficulty of precise quantification with sequence-specific probes (involving competition with DNA strands that complement the probe target, background problems with membranes, and nonavailability of sensitive measurements of bound probes) still remains. The introduction of real-time PCR technology has significantly improved and simplified the quantification of nucleic acids as described in Chap. 2.

One of the earliest clinical uses of PCR was in diagnosis of infectious diseases. Even with refinements, PCR still requires longer time than what is ideal for rapid diagnosis of infections in the point-of-care setting. Several non-PCR methods are fulfilling the need for rapid and simple diagnostics. For infectious agents such as HIV with substantial genetic divergence among strains, PCR (e.g., the TaqMan assay) becomes unfeasible and alternative detection methods such as microarrays and bead technologies may be required for sensitivity, speed, and cost.

Real-Time PCR Systems

Some of the limitations of end-point PCR have been addressed in real-time PCR systems, a number of which are now on the market. These systems offer many general technical advantages, including reduced probabilities of variability and contamination, as well as online monitoring and the lack of need for post reaction analyses. There are currently five main chemistries used for the detection of PCR product during real-time PCR: DNA binding fluorophores, 5′ endonuclease, adjacent linear and hairpin oligoprobes, and the self-fluorescing amplicons. Some of real-time PCR systems were developed with contemporary applications such as quantitative PCR, multiplexing, and high-throughput (HT) analysis in mind. In real-time PCR the amount of product formed is monitored during the course of the reaction by monitoring the fluorescence of dyes or probes introduced into the reaction that is proportional to the amount of product formed, and the number of amplification cycles required to obtain a particular amount of DNA molecules is registered. Assuming a certain amplification efficiency, which typically is close to a doubling of the number of molecules per amplification cycle, it is possible to calculate the number of DNA molecules of the amplified sequence that were initially present in the sample. With the highly efficient detection chemistries, sensitive instrumentation, and optimized assays that are available today the number of DNA molecules of a particular sequence in a complex sample can be determined with unprecedented accuracy and sensitivity sufficient to detect a single molecule.

Typical uses of real-time PCR include pathogen detection, gene expression analysis, single nucleotide polymorphism (SNP) analysis, analysis of chromosome aberrations, and most recently also protein detection by real-time immuno PCR. Real-time quantitative PCR is a highly sensitive method that is especially useful for evaluating RNA fingerprints obtained from siRNA experiments and for scientists using RNAi for mapping cellular pathways. Applications of PCR in RNAi are discussed in a special report (Jain 2017a).

Several real-time PCR systems are available commercially. Each of these systems employs either one of several general types of fluorescent probes for detection – TaqMan probe, molecular beacons and SYBR Green 1 DNA-binding dye. LightCycler is the best known of real-time PCR systems.

Limitations of Real-Time PCR

Limitations of real-time PCR include that PCR product increases exponentially and variation increases with cycle number. There is an increased variation after transformation to linear values and increased risk of false negative results.

When performing standard-curve gene quantitation, each gene requires a standard curve, which takes up a lot of space on the standard 96-well plate. However, with the 384-well format of the AB7900, this is less of a problem. The need for a plasmid, oligonucleotide, or other source for the standard curve is an extra requirement and can lead to variation, making it difficult to compare data from different plates. When using the standard-curve method, an additional source of error can be introduced at the RT step as different RNA samples may have variable RT efficiencies. In spite of these limitations, the standard-curve method is still a useful method.

Future Applications of Real-Time qPCR

Of the important applications is the combination of real-time PCR with either laser capture microdissection or nucleic acids from paraffin-fixed archival samples or whole-transcript amplification from very small numbers of cells. It will be possible to measure gene expression or DNA copy number in specific cell types that are available only in a small quantity. Real-time qPCR can be applied to analysis of clinical samples to help stratification of patients in personalized medicine approach. The safety of cell-derived biological compounds or quantification of retrovirus-like particles will be enhanced with real-time qPCR. It will also be useful for identification of potential contaminants during the production of recombinant monoclonal antibodies (MAbs) for therapeutic use. Combining techniques for sorting fetal cells or DNA from the maternal circulation with qPCR will enable early minimally invasive prenatal diagnosis of numerous congenital disorders. Confirmation of expression levels of selected genes from microarray experiments will continue to be conducted using real-time qPCR methods.

Real-Time qPCR for Quantification of Circulating mtDNA

Circulating mtDNA is a potential biomarker of cellular mitochondrial dysfunction, which occurs in several human diseases. Changes in mtDNA are usually determined by quantification of mtDNA relative to nuclear DNA (mt/nDNA) using real-time qPCR. methodology and A study has identified that current methods for measuring mt/nDNA need to be improved as they have at least one of the following 3 problems: (1) as much of the mitochondrial genome is duplicated in the nuclear genome, many commonly used mtDNA primers co-amplify homologous pseudogenes found in the nuclear genome; (2) use of regions from genes such as β-actin and 18S rRNA, which are repetitive and/or highly variable for qPCR of the nuclear genome leads to

errors; and (3) the size difference of mitochondrial and nuclear genomes cause a dilution bias when template DNA is diluted (Malik et al. 2011). These authors described a qPCR-based method using unique regions in the human mitochondrial genome not duplicated in the nuclear genome; unique single copy region in the nuclear genome and template treatment to remove dilution bias, and to accurately quantify mtDNA from human samples.

Combined PCR-ELISA

PCR is limited with respect to gene quantification because of the exponential amplification of DNA, the tendency to occasionally amplify non-specific DNA, and the semi-quantitative character of such common DNA measurement techniques as Southern blotting or densitometry. Combined PCR-ELISA CR has been developed by scientists for overcoming the limitation of for quantitative gene analysis. This method combines certain features of PCR and enzyme-linked immunoassay (ELISA) techniques for accurate, high-precision measurements of hybridization with sequence-specific probes. Unlike Southern blotting, the combined PCR-ELISA enables complete removal of competing DNA strands prior to hybridization and does not have the background problems associated with membranes. The quantification of the input DNA using this method is independent of the number of PCR amplification cycles, and can be calculated automatically with a standard laboratory ELISA plate reader. With these advances over current technology, the gene quantification method should find wide application in a variety of diagnostic and research applications in clinical chemistry, microbiology, and genetics. Possible applications include:

- Diagnosis and typing of infectious diseases
- HLA typing for organ transplantation and autoimmune disease diagnosis
- Cancer diagnosis by oncogene detection
- Diagnosis of genetic diseased
- Monitoring of inflammatory diseases by assaying cytokine gene expression

Non-PCR Methods

A number of non-PCR technologies for nucleic acid amplifications have emerged that offer advantages for particular applications. The advantages include added convenience and cost-effectiveness as no thermal cycler is needed. They often require minimal sample preparation as they rely on a series of steps to add reagents. Many of these are complementary rather than competitive to PCR. Some of these technologies are described in the following text.

Linked Linear Amplification

Linked Linear Amplification (LLA) is a new nucleic acid amplification method that uses multiple cycles of primer extension reactions. The presence of nonreplicable elements in LLA primers renders primer extension products unusable as templates for further amplification, leading to linear accumulation of products. Through the use of nested primers, linear reactions can be "linked", providing total amplification yields comparable to those obtained by PCR. The LLA model predicts (a) that amplification yield will approach that of PCR as the number of primers increases and (b) that the unique composition of LLA products will give lower carryover amplification efficiency compared with PCR. LLA is a robust target amplification method with an advantage over PCR that it is more resistant to false results caused by carryover amplicon contamination.

Transcription Mediated Amplification

Transcription mediated amplification is an isothermal nucleic-acid-based method that can amplify RNA or DNA targets a billionfold in less than one hour's time. This system is useful for detecting the presence of *M. tuberculosis* and *C. trachomatis*.

Developed at Gen-Probe, transcription mediated amplification technology uses two primers and two enzymes: RNA polymerase and reverse transcriptase. One primer contains a promoter sequence for RNA polymerase. In the first step of amplification, this primer hybridizes to the target rRNA at a defined site. Reverse transcriptase creates a DNA copy of the target rRNA by extension from the 3′ end of the promoter primer. The RNA in the resulting RNA:DNA duplex is degraded by the RNase activity of the reverse transcriptase. Next, a second primer binds to the DNA copy. A new strand of DNA is synthesized from the end of this primer by reverse transcriptase, creating a double-stranded DNA molecule. RNA polymerase recognizes the promoter sequence in the DNA template and initiates transcription. Each of the newly synthesized RNA amplicons reenters the transcription mediated amplification process and serves as a template for a new round of replication. The amplicons produced in these reactions are detected by chemiluminescence in a specific gene probe in hybridization protection assay.

Rapid Analysis of Gene Expression

Current techniques for analysis of gene expression either monitor one gene at a time, for example northern hybridization or RT-PCR methods, or are designed for the simultaneous analysis of thousands of genes, for example microarray hybridization or serial analysis of gene expression. To provide a flexible, intermediate scale

alternative, a PCR-based method RAGE (rapid analysis of gene expression) has been developed which allows expression changes to be determined in either a directed search of known genes, or an undirected survey of unknown genes. A single set of reagents and reaction conditions allows analyses of most genes in any eukaryote. The method is useful for assaying on the order of tens to hundreds of genes in multiple samples. Control experiments indicate reliable detection of changes in gene expression 2-fold and greater, and sensitivity of detection better than 1 in 10,000. This technology has been applied to investigate the changes in gene expression in human cells following treatment with a carcinogen and to determine the changes in large numbers of genes in early stage breast cancer. These molecular "signatures" of cancer may then be used to determine which tumors are likely to be responsive to chemotherapy.

WAVE Nucleic Acid Fragment Analysis System

Transgenomics' proprietary WAVE Nucleic Acid Fragment Analysis System has been designed and optimized to use the DNASep cartridge for separating (ds or ss) DNA. WAVE is also commonly known as DHPLC (denaturing high performance liquid chromatography). The system can easily resolve PCR products that have small differences in their lengths. The WAVE System is based on the company's proprietary micro-bead technology. The patented micro-beads are packed into proprietary DNASep separation column, which is the key component of WAVE System. Each micro-bead has specific surface chemistry that interacts with DNA molecules. The DNA molecules are then selectively separated from the micro-beads with a mixture of liquid reagents. The system is computer controlled and contains the proprietary software WAVEMAKER, which predicts analytical parameters with a high accuracy for optimal fragment separation and mutational analysis. The WAVE HS System incorporates fluorescence detection to expand the high-sensitivity in analysis of nucleic acid fragments. Advantages include high sensitive, accuracy (can detect as little as 1 copy of a mutated allele in 100 wild type copies), speed, cost-effectiveness and versatility as it allows many fluorescent labels to be utilized. Because of this versatility, the WAVE System can essentially replace the use of traditional gel electrophoresis in the molecular biology laboratory. Applications include the following:

- Mutation screening
- Polymorphic marker mapping
- Linkage analysis
- Cancer epidemiology
- Population studies
- Forensic and paternity analysis (STR)
- Loss of heterozygosity (LOH)

WAVE analyzes previously identified genes for any variations, changes or mutations. Mutations discovered by DHPLC may provide researchers with critical information about the cause, onset and progression of certain diseases. Scanning for mutations in genes with the WAVE System relies on the specific binding of complementary strands of the DNA double helix. If a mutation exists, a DNA heteroduplex (pairing of different strands) is formed and the binding is less "tight." High temperatures can be used to denature (melt) the DNA double helix. If a mutation exists, the melting temperature of the heteroduplex will be lower. Partially melted DNA can be easily separated from unmelted DNA homoduplexes (pairings of similar strands) containing no mutation. WAVE technology is ideal for the design of new tests for inherited diseases, particularly those characterized by a variety of potential mutation sites dispersed across large or complex genes. Since the WAVE System detects any mutation within a particular DNA fragment, there is no need to design and optimize a specific probe- or primer-based assay for each individual mutation.

DNA Probes with Conjugated Minor Groove Binder

Minor groove binder (MGB)-oligonucleotides (ODN) conjugates technology, which form of extremely stable duplexes with single-stranded DNA targets, enables shorter probes to be used for hybridization based assays. In comparison with unmodified DNA, MGB probes have higher melting temperature and increased specificity, especially when there is a mismatch in the MGB region of the duplex. The fluorogenic MGB probes are more specific than standard DNA probes for single base mismatches and fluorescence quenching is more efficient, giving increased sensitivity, especially for single base mismatches at elevated hybridization temperatures. The company scientists are determining the utility of the MGB probe for other hybridization-based assays such as DNA microarrays.

Rolling Circle Amplification Technology

Rolling circle amplification technology (RCAT), an amplification process commercialized by QIAGEN, has significant advantages in terms of sensitivity, multiplexing, dynamic range and scalability. RCAT can achieve the following:

- Detect single target molecules or "analytes".
- Amplify signals from proteins as well as DNA and RNA.
- Pinpoint the location of molecules that have been amplified on a solid surface (in situ analysis/ biochips) since, unlike PCR, the amplified product remains attached to the target molecule.
- Measure many different targets simultaneously.
- Improve the ease and accuracy of quantitation.

- Simplify haplotype identification through phasing.
- Increase sensitivity with up to 10^{12} -fold amplification in one hour.
- Amplify DNA templates that vary in length from 1 base pair to over 100 Kilobases.
- Obviate the need for the time-consuming and expensive steps of thermal cycling currently.
- Analyze targets in solution or solid phase.

Because of its flexibility, sensitivity and reproducibility, RCAT is poised to play a significant role in clinical diagnostics and genetic analysis. The advantages of RCAT apply to a wide variety of analytes with relevance to virtually any disease.

Gene-Based Diagnostics through RCAT

Genetic materials can be measured directly by RCAT. The technology is ultra-sensitive and has the capability of detecting the presence of rare genetic mutations within otherwise normal tissues and cell populations. RCAT has been useful for detecting point mutations in isolated nucleic acids, but its application in cytological preparations has been problematic. By pretreating cells with a combination of restriction enzymes and exonucleases, RCAT in situ can detect gene copy number and single base mutations in fixed cells with efficiencies up to 90%. It can also detect and quantify transcribed RNA in individual cells, making it a versatile tool for cell-based assays. RCAT promises to greatly improve the performance of gene-based diagnostic testing and to facilitate the detection of a wide variety of infectious agents, cancerous cells, and genetic variances. RCAT offers a simple and rapid alternative for gene-based diagnostics, which can yield an amplification of signal from a target nucleic acid of a trillion-fold in about an hour without the need for thermal cycling.

RCAT is a highly flexible platform that can be performed in solution, in situ, and in microarrays and can be used for single-base discrimination as well as target quantitation. Furthermore, as RCAT is continuous, isothermal process, specialized thermal-cycling equipment is not needed. Finally, in contrast to target amplification methods such as PCR, which make an enormous amount of target nucleic acid sequences where once there was little, RCAT is essentially a signal amplification system so contamination of new reactions with previously amplified target sequences is not a concern. Therefore, the need to carry out the different parts of the amplification process in separate areas of the laboratory, a significant barrier for using PCR in a clinical setting, is eliminated.

RCAT-Immunodiagnostics

The application of RCAT in immunodiagnostics offers a significant opportunity for increasing the sensitivity of these tests. Some recent approaches to molecular diagnostics use mRNA measurement as a surrogate for direct protein measurement, but

changes in the concentration of an mRNA and its encoded protein are at times not proportional. In immuno-RCAT, a unique DNA sequence tag is associated with a particular antibody using a covalent linkage. Antibodies bound to antigen are then measured by RCAT-based amplification of the associated DNA tag.

Each antibody-bound DNA tag is amplified and the resulting long DNA product remains bound to the antibody-antigen complex during the entire reaction. The amplified product can then be collapsed to a point source; permitting ultrasensitive localized detection capability (the technology has been demonstrated to detect single antibody-antigen complexes). Immuno-RCAT is readily adaptable to high-throughput surface array formats and simultaneous multi-analyte detection systems.

RCAT-Biochips

RCAT is the ideal amplification method for biochips. No other practical amplification method can recognize, amplify and detect target molecules directly on a solid surface. Currently, gene arrays place spots or "elements" in a high-density matrix on slides. Each spot contributes a single piece of genetic information to the analysis. RCAT, from a single element in such an array, can obtain information on multiple analytes simultaneously, exponentially increasing the level of information available. Since RCAT can amplify and detect signal from a solid-phase target simultaneously, it provides the only means for a homogeneous biochip assay.

RCAT-Pharmacogenomics

RCAT adds significant value in the genomics area. It can be used to localize DNA sequences to particular sites on chromosomes, to amplify and isolate DNA sequences without cloning in vivo, and to identify single base-pair genetic differences between individuals. A simple method using RCAT can amplify vector DNA such as plasmid DNA from single colonies 10,000-fold in a few hours. MSI is developing biochips based on RCAT that perform ultra-high throughput SNP analysis (both genotyping and haplotyping) for identification of disease genes and drug response genes. Designs for RCAT SNP Chips include arraying thousands of distinct patient samples or tens of thousands of SNPs on a single biochip. RCAT is the only amplification method that can perform direct molecular haplotype analysis.

Circle-to-Circle Amplification

Circle-to-circle amplification is a tightly controlled process for strand-specific amplification of circularized DNA molecules. Tandem repeated complements of DNA circles are generated by rolling-circle replication, and converted to monomer

circles of opposite polarity to that of the starting material. These circles are then subjected to one more round of rolling-circle replication and circularization, and the process can be further repeated. The method can be directed to produce single-stranded circular or linear monomers, or linear concatemers of the desired polarity. The reaction is not product inhibited, and can yield 100-fold higher concentrations of monomer products than PCR. Each generation of the amplification process proceeds in a linear fashion, ensuring precise quantification. The procedure is suitable for parallel amplification of large numbers of DNA circles, because the few cycles and the robust reaction mechanism preserve the proportion of amplified molecules. This method can be used for multiplexed genotyping of polymorphic loci and for quantitative DNA analysis.

Biochips and Microarrays

Biochip is a broad term indicating the use of microchip technology in molecular biology and can be defined as arrays of selected biomolecules immobilized on a surface. DNA microarray is a rapid method of sequencing and analyzing genes. An array is an orderly arrangement of samples. The sample spot sizes in microarray are usually less than 200 microns in diameter. It is comprised of DNA probes formatted on a microscale plus the instruments needed to handle samples (automated robotics), read the reporter molecules (scanners) and analyze the data (bioinformatic tools). Hybridization of RNA or DNA-derived samples on chips allows the monitoring of expression of mRNAs or the occurrence of polymorphisms in genomic DNA In a DNA chip, an array of oligonucleotides or peptide nucleic acid probes is synthesized either in situ (on-chip) or by conventional synthesis followed by on-chip immobilization. The array is exposed to labeled sample DNA, hybridized, and the identity/abundance of complementary sequences is determined. For practical purposes, the terms “DNA microarray” and “DNA chip” are used as synonyms although there are some technical differences as already indicated.

A microarray is a collection of miniaturized test sites arranged on a surface that permits many tests to be performed simultaneously, or in parallel, in order to achieve higher throughput. The average size of test sites in a microarray and the spacing between them defines the array’s density. Higher density increases parallel processing throughput. In addition to increasing the throughput, higher density reduces the required volume for the sample being tested, and thereby lowers costs. Currently, the principal commercially available ways to produce microarrays include mechanical deposition, bead immobilization, inkjet printing and photolithography.

Applications of Biochips/Microarrays

Applications of biochip technologies in relation to biomarkers and molecular diagnostics are shown in Table 3.1.

Table 3.1 Applications of biochip/microarray technology in relation to biomarkers

Research applications
Study of gene expression in diseases
Discovery of biomarkers
Rapid DNA sequencing
Design and stratification of clinical trials
Drug safety applications: pharmacogenetics and toxicogenomics
Genetic screening and detection of single nucleotide polymorphisms (SNPs)
Identification of pathogens and resistance in infections
Molecular oncology
Cancer prognosis
Cancer diagnosis
Cancer typing
Detection of biological and chemical warfare agents
Pharmacogenomics
Gene identification
Genetic mapping
Gene expression profiling
Integration of diagnosis and therapeutics

Role of Biochip/Microarrays in Discovery of Biomarkers

The use of gene-expression microarrays in patient-based research creates new prospects for the discovery of diagnostic biomarkers and the identification of genes or pathways linked to pathogenesis. Application of this technology has been mostly in cancer and is described further in Chap. 6. Some noncancer applications are mentioned here.

The use of microarrays to analyze patient blood transcriptional profiles offers a means to investigate the immunological mechanisms relevant to human diseases on a genome-wide scale. Such studies provide a basis for the discovery of clinically relevant biomarker signatures. A strategy for microarray analysis was designed that is based on the identification of transcriptional modules formed by genes coordinately expressed in multiple disease data sets (Chaussabel et al. 2008). Mapping changes in gene expression at the module level generated disease-specific transcriptional fingerprints that provide a stable framework for the visualization and functional interpretation of microarray data. These transcriptional modules were used as a basis for the selection of biomarkers and the development of a multivariate transcriptional indicator of disease progression in patients with systemic lupus erythematosus.

Protein microarrays have been used for the discovery of novel disease biomarkers through antibody-based assays. A disease-specific antibody repertoire may be defined by immune response profiling. The antigens and antibodies revealed by suche studies are useful for clinical assay development, with enormous potential in applications for diagnosis, prognosis, disease staging and treatment selection.

Protein lysate microarray (LMA) technology is used for verification of presence and quantification of human tissue samples for protein biomarkers. Sub-picogram range sensitivity has been achieved on LMA using a non-enzymatic protein detection methodology. The optimized LMA methodology has been applied for verification of the presence and quantification of disease markers for atherosclerosis. LMAs have been used to measure apolipoprotein B100 in carotid endarterectomy samples. The data generated by LMA are validated by ELISA using the same protein lysates. The sensitivity, reproducibility, and high-throughput quality of LMA technology make it a potentially powerful technology for profiling disease specific protein markers in clinical samples.

Biomarkers and High Throughput Molecular Screening

High-throughput molecular screening tools have created a need for equally rapid means to verify potential biomarkers. ELISA microarray technology as a high-throughput system for cancer biomarker validation.

NIH's Molecular Libraries and Imaging program, which transitioned from the common fund in 2013 (https://commonfund.nih.gov/molecularlibraries/index), offers biomedical researchers access to the large-scale screening capacity necessary to identify small molecules that could be optimized as chemical probes to study the functions of genes, cells, and biochemical pathways in health and disease. They are also used by researchers in the public and private sectors to validate new drug targets, which could then move into the drug-development pipeline. The goal of the program is to initiate a continuously evolving stream of scientifically and technologically outstanding assays that can be miniaturized, automated and further used for screening small molecules within the Molecular Libraries Screening Centers Network. Appropriate assays may include, but are not limited to: biochemical or cell-based assays of activity measuring target interactions involving small molecules, peptides, or other biological molecules; cell-based assays measuring cell signaling or the activity of biosynthetic pathways; assays of cellular or molecular phenotypes; modulation of gene expression, including effects on transcription, translation or RNA splicing; assays measuring protein-protein interactions; assays involving mutant proteins associated with disease; and assays using model organisms such as yeast, *C. elegans*, and zebrafish.

Detection and Expression Profiling of miRNA

A key step toward understanding the function of the hundreds of microRNAs (miRNAs) identified in animals is to determine their expression during development. miRNAs regulate translation, but not the stability of mRNAs, and this partially explains why gene expression profiles based on mRNA analysis do not always correlate with

protein expression data. Several molecular diagnostic technologies have been used for expression profiling of miRNA including PCR, LNA and biochips.

The discovery of stable miRNA species circulating in blood has led to increased research focus on disease-related variations in serum and plasma miRNA expression and the possibility that such variations could serve as noninvasive biomarkers for disease. However, working with serum and plasma miRNA presents various challenges in purification and characterization. Purification of circulating miRNA from serum or plasma can be performed using the miRNeasy Serum/Plasma Kit (QIAGEN) followed by real-time RT-PCR, which is a reliable technique for miRNA quantification.

Real-Time PCR for Expression Profiling of miRNAs

Northern blotting is currently the gold standard for miRNA detection but it is inherently insensitive. The precursor expression profile of miRNAs determined by the real-time PCR assay is identical to the mature miRNA expression profile determined by northern blotting. PCR assays can quantify miRNA precursors using SYBR green detection and may be scaled up to include all of the known human miRNA genes and can easily be adaptable to other organisms such as plants, *Caenorhabditis elegans* and Drosophila. A further study provides. PCR assays can be used to amplify and quantify mature miRNAs and studying the relationship between the levels of precursor and mature miRNAs in normal development and in human diseases such as cancer.

It is possible to analyze miRNA in single cells by using a real-time PCR-based 220-plex miRNA expression profiling method. Development of this technique will greatly facilitate miRNA-related research on cells, such as the founder population of primordial germ cells where rapid and dynamic changes occur in a few cells, and for analyzing heterogeneous population of cells. In these and similar cases, the method of single cell analysis is critical for elucidating the diverse roles of miRNAs.

Use of LNA to Explore miRNA

Several human miRNA genes are located near cancer-associated genomic regions. Disturbances of miRNA expression are observed in human cancers and many miRNAs may be useful as biomarkers for disease. Detection of these by LNA (Locked Nucleic Acids) probes and LNA-based assays is being carried out by Exiqon. Because LNA is a bicyclic high affinity RNA mimic with the sugar ring locked in the 3′-endo conformation, single mismatches give greater perturbation of Tm compared to standard nucleic acids. Up to 10-fold improvement of miRNA detection can be achieved on Northern blots by LNA probes. LNA probes also show excellent specificity for miRNA targets. This is the basis of Exiqon's mercury miRNA

analysis product. In situ detection of miRNAs in the animal models has been performed using LNA-modified DNA probes.

Microarrays for Analysis of miRNA Gene Expression

The size and low expression levels of miRNAs make their analysis difficult by the methods described above. Microarrays can be used to obtain highly reproducible results that reveal tissue-specific miRNA expression signatures, which can be confirmed by assessment of expression by Northern blots, real-time RT-PCR, and literature search. This approach is useful for the analysis of normal and disease states. Basic analysis of miRNA genes in tissues of mice, both adult and embryos at various stages, using semiquantitative approaches reveals that miRNAs show tissue-restricted and developmentally restricted expression patterns. Newer microarray formats in development include many brain- and embryonic stem cell-specific genes. Affymetrix offers Genisphere's miRNA reagents for use with its GeneChip miRNA 2.0 Arrays to identify miRNA biomarkers and develop new diagnostics.

NCode™ Array (Life Technologies Corp) enables sensitive profiling of miRNAs while using extremely simple methods that provide both experienced and novice microarray users a rapid path to new discoveries. NCode enables study miRNA function by profiling the miRNA expression patterns in a given disease or developmental state. This can be used in molecular diagnostics and drug discovery. Sensitive microarrays to survey full sets of miRNAs in neural stem cells during differentiation, both in culture and in vivo, enable detection of novel patterns of regulated miRNAs systematically. An understanding of these unexpected regulatory mechanisms provides novel targets for potentially managing or controlling differentiation in stem cells prior to therapeutic transplantation.

mirVana™ System (ThermoFisher Scientific) is used for miRNA microarray profiling. It involves isolation of total RNA, extraction and labeling of miRNA, and this is followed by hybridization and analysis. miRNA arrays to speed up biomarker discovery and unique biomarkers have been identified for various diseases. Role of aberrant miRNA gene expression is described in Chap. 13 along with biomarkers of cancer.

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Chapter 4
Biomarkers for Drug Discovery and Development

Introduction

Among the current applications of biomarkers those for drug discovery and development are one of the most important. Biomarkers are used in new approaches to remedy the shortage of new drugs and to the current lengthy and expensive process of drug development. Biomarkers can be used to predict and confirm target binding, to determine mechanism of action of a drug, pharmacokinetics, toxicity, and to monitor disease status, stratify patients, and determine treatment efficacy in clinical trials. The parallel role of biomarkers in the drug discovery process is shown in Fig. 4.1.

For biomarkers to assume their rightful role, greater understanding of the mechanism of disease progression and therapeutic intervention is required. Biomarkers should be considered while the therapeutic target is still being identified and the concept is being formulated. Exploratory biomarkers lay the groundwork for probable or known valid biomarkers and are useful for hypothesis generation. They fill in the gaps created by uncertainty of disease targets and variability in drug response. Institution of biomarker strategies early in drug development will facilitate the processes for bridging from preclinical to clinical development. These include identifying the biomarkers that will enable compound profiling, the clinical decision tree algorithms, and developing and validating the assays that will measure them. Biomarker-based drug development also integrates pharmacogenetics, pharmacogenomics, pharmacoproteomics, and metabolomics which are relevant to the development of personalized medicine (see Chap. 18).

Biomarker Technologies for Drug Discovery

Several biomarker technologies are used for drug discovery. Those based on proteomics, and metabolomics will be discussed briefly in this section. The trend is to integrate various "-omics" technologies for drug discovery.

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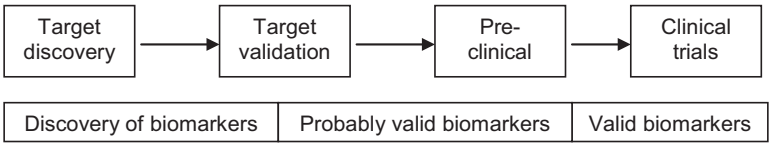

Fig. 4.1 Role of biomarkers in drug discovery and development process (© Jain PharmaBiotech)

Proteomics-Based Biomarkers for Drug Discovery

Use of proteomic technologies for discovery of biomarkers was described in Chap. 2. Here it will be extended to applications for drug discovery. Although biomarker discovery and drug discovery are considered separately, the two applications merge in case of proteomics. Biomarkers identified through proteomics can be used directly for drug discovery because proteins represent the majority of drug targets. Proteomic studies can focus on previously unrecognized pathways that may be targetable by drugs. As the drug development process proceeds further from discovery to clinical trials, the parallel process of biomarker validation and translation is important.

Some of the challenges of biomarker discovery and validation are due to complexity of the human proteome as numerous proteins may derive from a single gene sequence by over 300 posttranslational events. For the drug discovery phase common to biomarker and drug target identification, robust technologies are available for protein separation and identification. 2D–GE and LC-MS/MS are complementary technologies. The translation of biomarkers from discovery to the clinic also requires the development of immunochemical methods for performing validation studies followed by multiplexed assays that can be applied in a high throughput routine.

Chemoproteomics

Chemoproteomics or chemical proteomics is complementary to chemical genomics and involves the use of proteomic approaches to study how small molecules interact with cells. It also deals with "chemome" which is the non-enzymatic, chemical modifications of biomolecules in the body. Chemical proteomics has been applied to target identification and drug discovery.

Activity-Based Chemical Proteomics

Activity-based chemical proteomics is an important approach for chemical proteomics and was established in an attempt to focus proteomic efforts on subsets of physiologically important protein targets. Traditional proteomics methods enable

global analysis of protein abundance but do not provide information on the regulation of protein activity. Proteases, in particular, are known for their multilayered post-translational activity regulation that can lead to a significant difference between protease abundance levels and their enzyme activity. Activity-based proteomics can characterize protein activity and monitor the functional regulation of enzymes in complex proteomes.

Activity-based approach to proteomics is centered around the use of small molecules termed activity-based probes (ABPs) as a means to tag, enrich, and isolate, distinct sets of proteins based on their enzymatic activity. ABPs contain three key features; (1) a 'warhead', which binds irreversibly but selectively to the active site; (2) a 'tag' that allows enzyme 'handling' with a combination of fluorescent, affinity and/or radio labels; and (3) a linker region between warhead and tag (Heal et al. 2008). ABPs function as highly specific, mechanism-based reagents that provide a direct readout of enzymatic activity within complex proteomes. Modification of protein targets by an ABP facilitates their purification and isolation, thereby eliminating many of the confounding issues of dynamic range in protein abundance. This technology can be applied to advance the fields of biomarker discovery, in vivo imaging, and small molecule screening and drug target discovery. Measuring enzymatic activities in biological fluids is a form of activity-based proteomics and may be utilized as a means of developing disease biomarkers (Villanueva et al. 2009). Activity-based assays allow amplification of output signals, thus potentially visualizing low-abundant enzymes on a virtually transparent whole-proteome background. Activity-based protein profiling (ABPP) has emerged as a powerful chemical proteomic strategy to characterize enzyme function directly in native biological systems on a global scale. Detection, visualization, and identification of active proteases can be facilitated by activity-based probes, which covalently bind to a catalytic residue of the target protease. Synthesis of activity-based probes can be challenging. A simple protocol has been described for probe synthesis based on standard solid phase peptide synthesis followed by capping of the N-terminus with a reactive electrophile as a warhead (Van Kersavond et al. 2017).

Transcriptomics for Drug Discovery

Transciptome analysis, a part of functional proteomics, can provide a large-scale survey of gene expression associated with the etiology of a human disease. The quantitative and qualitative readouts can provide increased power to identify novel drug targets or biomarkers indicative of drug safety or efficacy.

Microarray analyses have already become standard tools to study transcription levels and patterns in cells. Furthermore, advances in 2D GE and MS are providing new insights into the function of specific gene products. Full understanding of the proteome, however, requires more than gene expression levels as many proteins undergo post-translational modifications that dictate intracellular location, stability, activity and ultimately function. Relying exclusively on mRNA levels to measure

protein function can therefore be misleading, and thus requires additional information about protein levels and modifications as well as signaling pathways and metabolite concentrations and distribution. These large-scale approaches, aided by using bioinformatics to analyze the data, now generate more biological information than previously possible. Since the efficacy and safety of many drugs is related to multiple changes in gene expression, these findings are driving drug discovery and diagnostics into an age of multiple target programs and multiplexed measurements.

Multiplex biomarker tests are becoming an essential part of the drug development process. Biomarker-based tests as effective tools in improving preclinical research and clinical development, and the challenges that this presents. Incorporation of biomarkers in the clinical pipeline can improve decision making, accelerate drug development, improve translation, and reduce drug development costs (Rahmoune and Guest 2017).

Metabolomics for Drug Discovery

Application of metabolomics in the exploratory stages of drug discovery, particularly in combination with high throughput screening, improves compound/target selection and reduces costs. Lipomic profiling (see Chap. 2) is a particularly powerful tool for drug discovery and target validation because many common disorders such cardiovascular disease, obesity, and diabetes produce changes in lipid metabolism. Throughout the development process, lipomic profiling can be used to evaluate a drug's effect on metabolic pathways and to quickly identify good drug candidates. A lipomic profile can also eliminate seemingly effective drugs that disturb metabolic pathways to cause adverse effects. Lipomic profiling has the potential to identify toxic effects of drug compounds. Because toxins can alter lipid levels in serum or tissues, assaying for these changes could point out potential pitfalls early in the discovery process.

Biomarkers and Drug Safety

Biomarkers of Adverse Drug Reactions

Susceptibility to ADRs varies with genetic make up, age, sex, physiology, exogenous factors, and disease state. The clinical consequences of ADRs range from patient discomfort through serious clinical illness to the occasional fatality. Some facts about ADRs are:

- There are 2.2 million hospitalizations due to ADRs per year in the US.
- Fatal ADRs are the fourth leading cause of death in the US.
- ADRs are a serious problem in infants and children.

- ADRs are more frequent in the elderly – the fastest growing segment of the population in the US.
- Ethnic group may act as a marker for underlying genetic or environmental differences in the susceptibility to ADRs, e.g., during treatment with angiotensin converting enzymes and thrombolytic drugs.

The problems of ADRs in children is being increasingly recognized, and they differ from adult reactions in frequency, nature, and severity. Infants and young children, when exposed to some drugs such as anticholinergic agents, are more likely than adults to develop ADRs, but may also be less susceptible to toxic reactions to other drugs. ADRs in children caused by drugs of abuse are a major problem in the US. Children may be exposed to these drugs through in utero exposure during pregnancy, through breast feeding, and through exposure during adolescence. These ADRs can include effects on the nervous system, cognitive problems, cardiovascular anomalies, and, in the case of second-hand tobacco smoke, an increased risk for sudden infant death syndrome, acute respiratory infections, asthma, middle-ear disease, and multiple sclerosis in children.

In 2008, the US NIH announced funding to support research that includes use of genomics, proteomics, and transcriptomics technologies in the discovery and identification of toxicity biomarkers; use of metabolomics alone or in combination with other technology to identify and characterize novel toxicity-associated drug metabolites and unraveling of novel ADR mechanisms; genomic studies that may identify animals that develop idiosyncratic reactions similar to humans; using genomics to define patterns of genes association with pediatric ADRs; placental genomics, proteomics, and biomarker identification to understand ADRs; the role of epigenetic factors to explain or predict developmental differences in the expression of ADRs; and other studies.

Applications of Biomarkers in Drug Safety Studies

Examples of conventional biomarkers of drug toxicity are measurement of serum transaminases for liver toxicity, serum levels of urea nitrogen and creatinine for kidney toxicity, serum creatinine phosphokinase for muscle damage. These have generally been useful for detecting liver and kidney damage after it has occurred but cannot be used to identify patients at risk of developing side effects. Companies have hesitation in developing toxicity biomarkers as they are afraid that FDA will insist on their use, which might eliminate some drugs in development even though the value of the test may not be certain.

In the preclinical testing where toxicity is largely based on histopathology, noninvasive biomarkers appearing in body fluids can be used to develop interspecies bridging biomarkers that correlate well with minimal or mild histopathology based toxicity end points. Biomarkers of drug-specific mechanisms of toxicity can be incorporated to increase confidence in evaluating drug safety in clinical settings.

With better biomarkers to monitor early safety and toxicity in clinical trials, the traditional use of safety margins in clinical drug development can be improved. Several technologies described earlier in this report are used for drug safety studies at preclinical stage as well as during drug development. Various "omics" technologies are used in generating these biomarkers, particularly genomics, proteomics and metabonomics.

The Predictive Safety Testing Consortium (PSTC), one of nine consortia comprising the Critical Path Institute (C-Path), is a unique, public-private partnership that brings pharmaceutical companies together to share and validate safety testing methods with the advice of worldwide regulatory agencies, including the FDA, the European Medicines Agency, and the Japanese Pharmaceuticals and Medical Devices Agency. The corporate members of PSTC share a common goal: to find improved safety testing methods and approaches utilizing fluid-based safety biomarkers which accurately predict drug-induced tissue injury. Specifically, the primary goal of PSTC is the qualification of novel translational safety biomarkers for use in early clinical trials in order to enable safer investigations and development of new drug candidates. A publication has described the critical importance of improved safety biomarkers for the drug development process and the present state of the biomarker qualification process with regulatory agencies (Sauer et al. 2015). In addition, it highlights the work that the PSTC and its collaborative partners have done and continue to do to identify and qualify more selective and specific safety biomarkers. Finally, it describes ongoing efforts to better define the regulatory qualification process and an integrated translational safety strategy.

Genomic Technologies for Toxicology Biomarkers

Genomics can be applied in biomarker identification as a source of candidate markers or marker panels. In toxicology, the mechanism cannot yet be deduced from expression data alone; therefore, genomic data are best utilized to provide direction into areas of research that should be pursued in the laboratory. These "functional" or "localization" studies are critical in providing validated toxicology biomarkers. Despite an abundance of candidates identified with genomic and other technologies, there is a scarcity of biomarkers that are readily applicable and interpretable alongside routine clinical chemistry in optimizing lead molecules. To validate candidate biomarkers after functional or localization studies, they must be tested in internal lead optimization studies routinely run in pharmaceutical preclinical development. Future technologies should integrate all assay types into one format that measures different analytes simultaneously regardless of whether the biomarkers are nucleic acids, proteins or metabolites. Testing in lead optimization studies provides the positive and negative control data necessary to build experience and give toxicology biomarkers meaning, so that decisions can be made from their application.

Proteomic Technologies for Toxicology Biomarkers

Proteomics can be applied for the identification and confirmation of peripheral biomarkers for altered liver function after toxicant exposure. Serum troponins are described as biomarkers of myocardial infarction in Chap. 15. Troponins can also be used as biomarkers of drug-induced cardiac toxicity. Proteins such as Kim 1 and clusterins can be used as biomarkers of nephrotoxicity.

Metabonomic Technologies for Toxicology Biomarkers

Metabonomics studies demonstrate its potential impact in the drug discovery process by enabling the incorporation of safety endpoints much earlier in the drug discovery process, reducing the likelihood (and cost) of later stage attrition.

Global metabolic profiling (metabonomics/metabolomics) has shown particular promise in the area of toxicology and drug development. A metabolic profile need not be a comprehensive survey of composition, nor need it be completely resolved and assigned, although these are all desirable attributes. For the profile to be useful across a range of problems, however, it must be amenable to quantitative interpretation and it should be relatively unbiased in its scope. In addition to explicit quantification of individual metabolites, analytical profiles such as NMR spectra are effectively functions of the concentrations of the constituents of the sample and hence can be handled directly as metabolic profiles. A further requirement for the platform used to generate profiles is that the analytical variation introduced postcollection be less than the typical variation in the normal population of interest, so as not to reduce significantly the opportunity to detect treatment/group-related differences. Fulfilling this condition is very dependent on the actual system and question in hand and is probably best tested in each new application.

In both preclinical screening and mechanistic exploration, metabolic profiling can offer rapid, noninvasive toxicological information that is robust and reproducible, with little or no added technical resources to existing studies in drug metabolism and toxicity. Extended into the assessment of efficacy and toxicity in the clinic, metabonomics may prove crucial in making personalized therapy and pharmacogenomics a reality.

Integration of Genomic and Metabonomic Data to Develop Toxicity Biomarkers

Global profiling of changes in gene expression or metabolites is used to identify biomarkers for drug development. There is a need to ‘validate’ the biomarker by linking it to a phenotypic change. However, it is difficult to connect an alteration in

mRNA levels, or of a metabolite that represents an endogenous product of intermediary metabolism, to a drug-induced toxicity. Therefore, if one can demonstrate that changes in gene expression correlate biochemically to a metabolic shift, the reliability using these endpoints as biomarkers is increased. Recent advances in annotation of metabonomic data and comprehensive metabolite pathway mapping tools increase the likelihood for successful "-omic" data integration.

Toxicology Studies Based on Biomarkers

Although substantial progress has been made on the identification of biomarkers and the establishment of screens derived from such toxicogenomics studies, there is still a shortage of biomarkers for important toxicity endpoints and for toxicology in general. The ultimate goal, of course, is predictive toxicogenomics, which is an attempt to infer the likelihood of occurrence of a toxic event with exposure to a new agent based upon comparative responses with large databases of gene, protein or metabolite expression data. Gene expression databases are currently limited by the fact that measurable toxic phenotypes generally precede or at best coincide with the earliest observable changes in transcriptional profiles.

It is important to detect toxicity of drugs by peripheral biomarkers before damage to various organs develops. Some pharmaceutical companies are beginning to use biomarkers for safety studies. An important goal involves using biomarkers to discover ways to identify toxicity as early as possible in animal studies. For example, Novartis has conducted animal studies for a number of compounds targeting inflammation and found that particular gene expression signatures in animal gastric tissue could predict gastrointestinal toxicity as early as day 1. Once they had identified the signature, they found that it also occurred in white blood cells, indicating that blood testing could be used as surrogate for gastrointestinal tract toxicity for this class of compounds. In a broader application, Novartis is also showing that organ-specific gene expression signatures can be used to detect the effects of compounds on various organs. Novartis routinely develops safety biomarkers for new compounds early in research. By knowing the target and looking at which organs are affected, researchers can more easily identify a compound's impact on the wrong organs. There are several examples where a drug that failed in early safety testing was identified to hit another target that could be useful for another disease. A CNS drug was dropped at Novartis because it induced apoptosis of gastric secreting cells in the stomach. It is now being explored as a potential treatment in some stomach diseases.

Toxicoproteomics seeks to identify critical proteins and pathways in biological systems that are affected by and respond to adverse chemical and environmental exposures using global protein expression technologies. Toxicoproteomics integrates three disciplinary areas: traditional toxicology and pathology, differential protein and gene expression analysis, and systems biology. Proteomics also facilitates discovery of new biomarkers and toxicity signatures.

Preclinical safety testing is an important part of drug development. The concern for safety continues into the marketed phase of the drug and adverse effects are recorded even though a pathomechanism is not always known. Benefits of using preclinical biomarkers of drug safety are:

- Reduction of the time and resources needed to screen new therapeutic candidates
- Improvement of the process of lead candidate selection in drug discovery
- Insights into mechanisms of toxicity
- Prediction of toxicity at an early stage

One approach for cost/time reduction is through development of early in vivo markers of toxic response. The integration of predictive biomarkers with traditional methods for drug safety testing may provide a stronger rationale for selecting compounds to advance into the clinic. Advances in overall protein and metabolic rofiling now make it possible to perform simultaneous toxicodynamic (TD) biomarker discovery, toxicokinetic analysis and drug metabolic profiling. Biomarkers are used to demonstrate a quantitative relationship between drug exposure and related pathological events. The toxicity biomarkers help to predict safety margins and guide dose selection. The broader potential of biomarker analysis however lies in clinical development and patient care. Biomarkers may be used to identify patient response and establish effective and safe dosage regimens. Biomarkers may also reveal on-target versus off-target response and toxicological outcomes during drug treatment.

Major organs affected by toxic effects of drugs are the liver, kidneys, brain and the heart. Examples of biomarkers of toxicity of these organs will be given in the following sections.

Biomarkers of Hepatotoxicity

The study of hepatotoxicity in vitro is complicated by the difficulty of maintaining hepatocytes in culture due to a lack of understanding of the humoral and matrix requirements of these cells. A variety of in vitro models of the liver have been developed, such as perfused livers, liver slices and three-dimensional perfused bioreactors, but the static cell culture is the most commonly used system. Technological advances in genomics, proteomics and metabonomics are playing a very important role in uncovering novel biochemical pathways and biomarkers of toxicity. Several of these studies have focused on hepatotoxicity, particularly on the effects of acetaminophen, carbon tetrachloride and aflatoxin B1.

Aviva Systems Biology is producing antibodies that the Institute for Systems Biology (ISB) uses to identify liver toxicity biomarkers. Aviva develops the antibodies from a list of proteins provided by the ISB. ISB will then assess the efficacy of these antibodies and will use them to find biomarker proteins that work as early liver toxicity biomarkers.

High throughput toxicological estimation is required for safety evaluation in the early stage of drug discovery. In this context, establishment of an in vitro screening system reflecting in vivo toxicity is demanded for earlier safety assessment. Scientists at Pfizer have investigated LDH release and mitochondrial respiration (WST-1 reduction assay) to detect cytotoxicity, morphological evaluation, and proteomics for estimating the reliable and sensitive biomarkers by using rat primary hepatocytes exposed to the compounds (acetaminophen, amiodarone, tetracycline and carbon tetrachloride) that are known to induce hepatotoxicity. They concluded that the cytotoxicity was detected earlier by measuring WST-1 than by measuring LDH release because the reduction of mitochondrial respiration is an expressions of earlier toxicity for cellular function, while the measured increase in the LDH release occurs after the failure of the cell membrane. Mitochondrial respiration ability was a useful parameter of cytotoxicity for in vitro hepatotoxicity screening, as cytotoxicity can be detected during the early stage of exposure. In addition to the conventional biomarkers, several protein biomarkers which relate to oxidative stress and metabolism-regulation were detected. Further comprehensive analysis of defined proteins would be necessary to estimate the more sensitive toxicology biomarker.

Valproic acid, which is a widely used antiepileptic drug, is associated with oxidative stress in rats, as demonstrated by elevated levels of 15-F(2t)-isoprostane, which precedes the onset of liver necrosis, steatosis, and elevated levels of serum alpha-GST. At Eli Lilly & Co, biomarkers are being used to predict hepatotoxicity from preclinical information. The company is working with FDA to develop biomarker standards that will enable the company to evaluate hepatotoxicity in the clinic.

Using acetaminophen overdose-induced liver injury in the mouse as a model system, highly significant differences have been observed in the spectrum and levels of miRNAs in both liver tissues and in plasma between control and overdosed animals (Wang et al. 2009). Specific miRNA species, such as mir-122 and mir-192, are enriched in the liver tissue and exhibit dose- and exposure duration-dependent changes in the plasma that parallel serum aminotransferase levels and the histopathology of liver degeneration, but their changes can be detected significantly earlier. These findings suggest the potential of using specific circulating miRNAs as sensitive biomarkers for drug-induced liver injury.

In 2011, Critical Path Institute (Tucson, Arizona) and The Hamner Institutes for Health Sciences, both non-profit research organizations, started collaboration to find biomarkers for monitoring drug-induced liver injury. They will focus on regulatory science research for predicting, detecting, and monitoring liver safety issues during the development of new medicines. The goal is to generate data and reach scientific consensus for biomarkers and other methods that would be submitted to the FDA to be qualified for specific uses in product development.

A systematic strategy has been used for drug-induced hepatotoxicity to establish specific screening biomarkers and these were applied to evaluate whether the drugs have potential hepatotoxicity in rat models (Li et al. 2016). Fifteen common biomarkers were screened by multivariate statistical analysis and integration analysis-based metabolomics data. The receiver operating characteristic (ROC) curve was used to evaluate the sensitivity and specificity of the biomarkers. The researchers

obtained 10 specific biomarker candidates with an area under the curve >0.7. Then, a support vector machine (SVM) model was established by extracting specific biomarker candidate data from the hepatotoxic drugs and nonhepatotoxic drugs; the accuracy of the model was 94.9% and the results demonstrated that those 10 biomarkers are specific. Six drugs were used to predict the hepatotoxicity by the SVM model; the prediction results were consistent with the biochemical and histopathological results, demonstrating that the model was reliable. Thus, this SVM model can be applied to discriminate the between the hepatic or nonhepatic toxicity of drugs. This approach not only presents a new strategy for screening-specific biomarkers with greater diagnostic significance but also provides a new evaluation pattern for hepatotoxicity, and it will be a highly useful tool in toxicity estimation and diagnosis of diseases.

Biomarkers of Nephrotoxicity

Most of pharmacological compounds and their metabolites are excreted via the urine, and within the complex structure of the kidney, the proximal tubules are a main target site of nephrotoxic compounds. Scientists at Amgen have used the model for nephrotoxicants mercuric chloride, 2-bromoethylamine hydrobromide, hexachlorobutadiene, mitomycin, amphotericin, and puromycin to elucidate time- and dose-dependent global gene expression changes associated with proximal tubular toxicity. Their data indicate that valid predictions could be made based on gene expression changes from a small set of expression profiles. A set of potential biomarkers showing a time- and dose-response with respect to the progression of proximal tubular toxicity were identified. These include several transporters (*Slc21a2, Slc15, Slc34a2), Kim 1, IGFbp-1, osteopontin, alpha-fibrinogen, and Gstalpha*.

Renal gene expression profiling coupled with analysis of classical end points affords promising opportunities to reveal potential new mechanistic biomarkers of renal toxicity including KIM-1, osteopontin and vimentin. An example of use of proteomic is investigation of dose-related nephrotoxicity caused by cyclosporine A, which has proven beneficial effects in organ transplantation. Proteomic analysis using 2D GE has demonstrated an association between calbinden-D 28 and cyclosporine A (CsA)-induced nephrotoxicity and is considered to be a biomarker for this adverse effect. This shows that proteomics can provide essential information in mechanistic toxicology. 2D GE and NMR spectroscopy has been used to study nephrotoxicity in the rat following exposure to puramycin aminonucleoside. Monitoring of proteins in the urine enabled a more detailed understanding of the nature and progression of the proteinuria associated with glomerular nephrotoxicity than was previously possible.

In studies on experimental animals treated with nephrotoxic or non-nephrotoxic compounds, biomarkers consisting of gene signatures based on gene expression can predict the future development of renal tubular degeneration weeks before it occurs. By comparison, histopathology or clinical chemistry fails to predict the future development of tubular degeneration, thus demonstrating the enhanced sensitivity of

gene expression relative to traditional methods for prediction of compound-induced pathology in the kidney.

Metabolomic experiments have been performed on Sprague-Dawley rats treated with the nephrotoxins gentamicin, cisplatin, or tobramycin to discover biochemical biomarkers useful for early identification of nephrotoxicity (Boudonck et al. 2009). Using a combination of gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry, a global, nontargeted metabolomics analysis was performed on urine and kidney samples collected after 1, 5, and 28 dosing days. Increases in polyamines and amino acids were observed in urine from drug-treated rats after a single dose, and prior to observable histological kidney damage and conventional clinical chemistry indicators of nephrotoxicity. Thus, these metabolites are potential biomarkers for the early detection of drug-induced nephrotoxicity. Upon prolonged dosing, nephrotoxin-induced changes included a progressive loss of amino acids in urine, concomitant with a decrease in amino acids and nucleosides in kidney tissue. A nephrotoxicity prediction model, based on the levels of branched-chain amino acids in urine, distinguished nephrotoxin-treated samples from vehicle-control samples, with 100%, 93%, and 70% accuracy at day 28, day 5, and day 1, respectively. Thus, this panel of biomarkers may provide a noninvasive method to detect kidney injury long before the onset of histopathological kidney damage.

KIM-1 measurements may facilitate sensitive, specific and accurate prediction of human nephrotoxicity in preclinical drug screens. Use of urinary trefoil factor 3 (TFF3) and albumin enables more sensitive and robust diagnosis of acute renal tubular injury than traditional biomarkers. To address the need for biomarkers that monitor recovery from agent-induced renal damage, changes were scored in the levels of urinary biomarkers in rats during recovery from renal injury induced by exposure to carbapenem A or gentamicin (Ozer et al. 2010). All biomarkers responded to histologic tubular toxicities to varied degrees and with different kinetics. After a recovery period, all biomarkers returned to levels approaching those observed in uninjured animals. In this study, an assay for serum cystatin C was found to be more sensitive and specific than serum creatinine or blood urea nitrogen in monitoring generalized renal function after exposure of rats to eight nephrotoxicants and two hepatotoxicants. This sensitive serum biomarker will enable testing of renal function in animal studies that do not involve urine collection.

Diagnostic potential of monocyte chemoattractant protein-1 (MCP-1), a protein that plays a role in recruiting immune cells to injured or infected sites in the body, has been assessed in acute kidney injury (AKI). Investigators found elevated levels of MCP-1 as well as its mRNA in urine samples from mice and humans with AKI, indicating that it can be used as a biomarker (Munshi et al. 2011).

Cardiotoxicity

Cardiotoxicity is a particular problem with some anticancer drugs. Biomarkers for cardiac safety that are employed in an increasing number of clinical programs designed for investigational oncology therapeutics: (1) assessment of left

ventricular ejection fraction by either echocardiography or multigated acquisition scan; and (2) electrophysiological measurement of QT/QTc duration, assessed by electrocardiogram, for predicting risk of a potentially fatal arrhythmia called torsades de pointes.

Anthracycline-induced cardiotoxicity can cause serious health problems for an increasing number of children surviving childhood malignancies. Early detection of cardiac failure is critically important for the prevention and management of anthracycline-induced cardiotoxicity. A review of studies of the use of the biomarkers B-type natriuretic peptide (BNP), N-terminal pro-BNP (NT-pro-BNP), cardiac troponin T (cTnT), and cardiac troponin I (cTnI) in relation with anthracycline-induced cardiotoxicity in children showed a significant relation between elevated biomarkers BNP, NT-pro-BNP, and cTnT and cardiac dysfunction (Mavinkurve-Groothuis et al. 2008). These might be useful biomarkers for the early detection of anthracycline-induced cardiotoxicity.

Patterns of proteomic signatures obtained from high dimensional mass spectrometry data have been used for analysis of serum from rat models for detection of anthracycline and anthracenedione induced cardiotoxicity. Diagnostic proteomic patterns have a potential for clinical utility because low molecular weight peptides and protein fragments may have higher accuracy than traditional biomarkers of cardiotoxicity such as troponins. These fragments may 1 day be harvested by circulating nanoparticles designed to absorb, enrich and amplify the diagnostic biomarker repertoire generated even at the critical initial stages of toxicity.

Neurotoxicity

Neurotoxicant-induced changes in protein level, function or regulation could have a detrimental effect on neuronal viability. Direct oxidative or covalent modifications of individual proteins by various chemicals or drugs are likely to lead to disturbance of tertiary structure and a loss of function of neurons. The proteome and the functional determinants of its individual protein components are, therefore, likely targets of neurotoxicant action and resulting characteristic disruptions could be critically involved in corresponding mechanisms of neurotoxicity. A variety of classic proteomic techniques (e.g. LC)/tandem mass spectroscopy, 2DG image analysis) as well as more recently developed approaches (e.g. two-hybrid systems, antibody arrays, protein chips, isotope-coded affinity tags, ICAT) are available to determine protein levels, identify components of multiprotein complexes and to detect post-translational changes. Proteomics, therefore, offers a comprehensive overview of cell proteins, and in the case of neurotoxicant exposure, can provide quantitative data regarding changes in corresponding expression levels and/or post-translational modifications that might be associated with neuron injury.

hESC-based in vitro screening models have been used for assessing embryonic neurotoxicity. These in vitro test systems are based on neuronal differentiated murine ESCs and quantitative differential proteomic display techniques to identify biomarkers for neurotoxicity. Results are superior to those of conventional array

technologies (nucleic acids), because the proteomic analysis covers posttranslational modifications. It is possible to identify toxicity biomarkers without using animal-based in vitro or in vivo systems.

To circumvent serious neurotoxicity, while taking advantage of the antitumor activities of the platinum agents, efforts to identify mechanism-based biomarkers are under way. These data from study of genetic biomarkers associated with neurotoxicity induced by single-agent and combination platinum chemotherapy have the potential for broad clinical implications if mechanistic associations lead to the development of toxicity modulators to minimize the noxious sequelae of platinum chemotherapy (McWhinney et al. 2009).

Applications of Biomarkers for Drug Development

Biomarkers and assays based on biomarkers are being used increasingly in the drug development process. The following applications of biomarkers are relevant to drug development (Salter and Holland 2014):

- Predictive biomarkers can be used to matching the most appropriate therapy to the mechanism of action for optimal benefit and to identify patients at risk of particular adverse events.
- Disease activity biomarkers allow an assessment of the current severity of disease and therefore, response to treatment.
- Drug effect biomarkers indicate target engagement and the generation of pharmacological effect of a drug. These biomarkers are useful for determining the required dose and regimen for a new medicine where effects in humans can be compared with effects in the preclinical experiments on disease models.
- Drug kinetics biomarkers are genetic variants in drug metabolizing enzymes and drug transporters to indicate causes of adverse drug effects and/or lack of drug efficacy.

Use of surrogate biomarkers was described in Chap. 1. The focus of here will be on the use of biomarkers in clinical trials.

Application of Metabonomics/Metabolomics for Drug Development

Metabolic profiling of body fluids and tissues by spectroscopic detection of endogenous and drug related metabolites enables the extraction of comprehensive biochemical information, which is of diagnostic and prognostic value. The power of the method relies on three basic properties: (1) there is almost no sample preparation involved; (2) spectroscopic methods, especially NMR, are extremely reproducible;

and (3) the nature of the data reflects actual biological events (physiological phenotype). Metabonomic analyses of body-fluids can be used in early drug development not only for predicting general organ toxicity as described in a preceding section but also for monitoring more specific effects such as phospholipidosis, peroxisome proliferation, changes of the steroidal biosyntheses, and changes of the gut microflora. Apart from monitoring changes of single biomarkers, simultaneous regulations of several metabolites within pathways are investigated. Comparing these changes with a database of metabonomic studies allows identifying studies with similar mechanistic toxicity. It is also demonstrated how metabonomics can be used for identifying new biomarkers, which allow drawing conclusions about mechanistic effects.

Novel and highly relevant biomarkers generated by innovative metabolomics application platform enables pharmaceutical and biotech companies to develop more efficacious and safer compounds as well as reduce time to market. Furthermore, this technology empowers diagnostic companies and hospitals to define and validate novel biomarkers for diagnosis of latent diseases before first symptoms surface. The ultimate objective is to improve the patient's quality of life through earlier disease detection, therefore, avoiding the need for costly and risky interventions.

Metabolon has used metabolomics to analyze human plasma yielding biomarkers that enables sorting of patients into clinically recognized subsets. These biomarkers are not only diagnostic, but will also provide insights into the underlying pathology and new targets and approaches for drug development.

Role of Pharmacokinetic/Pharmacodynamic Biomarkers in Drug Development

Appropriate pharmacokinetic (PK)/pharmacodynamic (PD) biomarkers facilitate proof-of-concept demonstrations for target modulation; enhance the rational selection of an optimal drug dose and schedule; and decision making such as whether to continue or discontinue a drug development project. In addition measurement of PK/PD biomarkers can minimize the uncertainty associated with prediction of drug safety and efficacy as well as reduce the high rate of drug attrition during development.

Pharmacodynamic (mechanism of action) biomarkers can be used to measure acute target inhibition in vivo and potentially for interpreting dose response relationships. Some examples are as follows:

1. Candidate RNA transcripts, identified and qualified by using quantitative PCR, can be used as potential pharmacodynamic markers for a selective inhibitor of T cell receptor signaling.
2. A computational approach is used at Merrimack Pharmaceuticals to rationally identify pharmacodynamic biomarkers that are the most sensitive indicators of insulin-like growth factor-1 receptor (IGF1-R) antagonism at the molecular level. A detailed mathematical model of the IGF signaling pathway is trained

with experimental data, and validated by comparing the predicted activity of a novel IGF1-R antagonist on downstream ERK and AKT phosphorylation to in vitro experimental results. Using the validated model, a portfolio of sensitivity analyses and simulations is generated that highlights the key proteins regulating the IGF pathway and identifies the biomarkers that characterize the efficacy of the antagonist.

3. Scientists at Bristol-Myers Squibb have predicted active drug plasma concentrations achieved in cancer patients by pharmacodynamic biomarkers. Using human tumor xenografts grown in nude mice, they have determined the in vivo pharmacodynamic response at efficacious doses of cetuximab – an anti-EGFR chimeric mouse/human MAb that has been approved for the treatment of advanced colon cancer (Luo et al. 2005). Three pharmacodynamic end points were evaluated: tumoral phospho-EGFR, tumoral mitogen-activated protein kinase (MAPK) phosphorylation, and Ki67 expression. A pharmacokinetic/pharmacodynamic model was established and predicted that the plasma concentration of cetuximab required to inhibit 90% of phospho-EGFR was 67.5 mug/mL. CONCLUSIONS: Phospho-EGFR/phospho-MAPK could be useful clinical biomarkers to assess EGFR inhibition by cetuximab.
4. Pharmacokinetic-pharmacodynamic correlations and biomarkers have been used for the development of COX-2 inhibitors. This solves a major problem in the development of COX inhibitors, where it is difficult to predict the appropriate dosing regimen for the treatment of chronic inflammatory pain, based upon information from preclinical studies and early clinical trials. Endogenous mediators of inflammation might be used as biomarkers for the analgesic effect and safety assessment. Such a biomarker can be an intermediate step between drug exposure and response. COX-2 inhibition, as determined by modelling of prostaglandin E2 (PGE2) levels in the whole blood assay in vitro can be used as a biomarker to predict drug effects (analgesia) in humans.
5. Pharmacodynamic biomarkers are used to measure receptor occupancy as a guide to the determination of optimal dose. Most direct estimate, however, is provided by ligand-displacement imaging using PET.

Molecular Imaging as a Biomarker in Drug Development

Imaging is now a recognized tool in drug discovery and development and is referred to as pharmaco-imaging. There is increasing evidence that human medical imaging can help answer key questions that arise during the drug development process.

Molecular Imaging in Preclinical Studies

Molecular imaging, using a variety of targeted probes and spanning optical magnetic resonance and PET methods, provides insights into target validation and disease mechanism issues that are crucial in preclinical studies. More traditional

imaging modalities, such as MRI, are used widely in animal studies. These preclinical studies take place in the context of high attrition rates for candidate pharmaceuticals when translating to human subjects, perhaps as a result of inadequate animal models of human pathophysiology. Ultimately, it is anticipated that greater use of imaging during pre-clinical stages will facilitate better translation from animal models to human subjects, by minimizing changes in experimental paradigms while the model organism is changed.

In preclinical studies, the greatest value of imaging analytics lies in early assessment of method of action. Whereas histology provides limited insight into the effects of a compound, image analysis demonstrates whether or not the drug behaves in the way that researchers predicted it would, e.g. does a compound bind to a particular receptor? Understanding of a disease or therapy may involve understanding the localization of biomarkers and not simply their measurement, for example, discovering where specific receptors are occupied or not. Ligand-displacement PET also provides a direct estimate of the receptor occupancy as a guide to determination of optimal dose. Having such questions answered at the preclinical stage enables optimization of clinical research from phase I through phase III.

To incorporate medical imaging into a study, two major decisions first must be made: (1) how to acquire the images; and (2) how to analyze the images once they have been acquired. As the use of medical imaging in preclinical research still is emerging, there is no established practice ready to handle image acquisition. Companies either outsource to facilities that offer imaging equipment and technicians to acquire the images for them, or pay for the use of a private facility and handle image acquisition themselves. For image analysis, companies can go one of two ways: they can hire the expertise internally, or outsource it to specialists who have the resources already in place. Automated analysis requires software experts to develop analysis algorithms and graphical user interface engineers, as well as provide staff to handle the database reporting tabulation and to conduct the analysis. Because of the breadth of expertise required, even large companies such as Pfizer, which have in-house imaging centers, have opted to outsource to companies that specialize in medical image analysis.

Researchers can take advantage of innovations in the development of image-based biomarkers from various US universities. The introduction of new biomarkers provides greater opportunity to study the mechanisms of action in a preclinical setting. One advantage here is that researchers' predictions can be verified through histology at the end of the study. However, imaging biomarkers are not easy to acquire or to extract and analyze. Full exploitation of such information requires access to a multidisciplinary team, including radiologists, physicists and software developers.

At Novartis investigation a PET tracer has been used to assess drug activity and mechanism of action for a CNS target in a psychiatric disease that is not well defined. A high concordance was found between tracer distribution and location of specific receptors. This outcome confirmed the drug activity and mechanism and supported assessment of receptor distribution in humans to support clinical development of the drug candidate.

Molecular Imaging in Clinical Trials

Imaging modalities such as MRI, CT and PET can offer significant insights into the bioactivity, pharmacokinetics and dosing of drugs, in addition to supporting registration applications. The best examples of the use of imaging are in clinical trials of anticancer drugs. Earlier indications might reduce a patient's exposure to ineffective, poorly tolerated, toxic or expensive treatments. Conversely, lack of bioactivity could represent sufficient cause for suspension or termination of further development of unpromising compounds.

Imatinib mesylate (Gleevec, Novartis) is a tyrosine kinase inhibitor that has gained FDA approval for chronic myelogenous leukemia and gastrointestinal stromal tumors (GIST). When using more-traditional methods, such as CT imaging for determining tumor size, to evaluate the effectiveness of imatinib on GIST, the first objective evaluation of tumor response is generally performed no earlier than 2–3 months after the start of treatment because earlier changes in tumor size are seldom significant. Using FDG–PET as an indicator of tumor metabolism, however, it was found that reduction in glucose metabolism preceded CT response by a median of 7 weeks, and all GIST patients with a complete or major metabolic response subsequently reached partial or durable stable disease on CT. In another cancer example, the overexpression of vascular endothelial growth factor (VEGF) is frequently implicated in tumor angiogenesis. In one trial of the VEGF-specific monoclonal antibody bevacizumab (Avastin, Genentech) on rectal cancer patients, CT measurements of tumor blood flow and blood volume decreased significantly 12 days after a single infusion of the drug. This observed decrease in tumor perfusion demonstrated a positive correlation with other tumor indicators, including microvessel density, interstitial fluid pressure, and circulating endothelial and progenitor cell levels.

Numerous past studies have shown that it is possible to derive the pharmacokinetic (PK) distribution of radiolabeled pharmaceuticals using PET imaging. In broad terms, imaging of PK properties falls into two categories. The first category involves the radiolabeling of compounds that interact with, or neutralize, agents from the environment, such as toxins, bacteria and viruses. In this case, generally only tissue concentrations of drugs are necessary. In the second category, if the drug is expected to alter or otherwise modulate some aspect of the pathophysiologic process, then imaging studies are generally used to characterize the number of receptors, binding efficiency and receptor occupancy. As an example of the first category, the development of 18F–labeled antifungal agent fluconazole (Diflucan, Pfizer) was monitored by PET to establish the concentration of the drug in different organs, particularly at the site of infection. The imaging study found that the observed concentrations compared favorably to the concentrations required to inhibit in vitro pathogen growth and provided valuable dosing information.

Quantitative MRI is playing an important role in an increasing proportion of clinical trials. This is particularly true in areas where both the disease and the effects of treatment manifest as structural or functional changes that can be directly imaged. MRI is, of course, less useful in areas such as pain management or psychological

illness, where the method offers little insight into biological processes, and in areas where convenient chemical biomarkers are available. One important example of an area where quantitative MRI can provide vital insight is the use of antiangiogenic or vascular disruptive agents in the treatment of solid tumors. Osteoarthritis is another area where quantitative MRI can have a major impact on study timelines, cost, and success. Differences among MRI scanner manufacturers and models in pulse sequence implementation, hardware capabilities, and even terminology make it increasingly difficult to ensure that results obtained at one center are comparable to those at another. A detailed MR protocol should be deployed a that is both effective and implementable across many different MRI systems and software versions (Ashton 2010).

Prospects of Molecular Imaging in Drug Discovery and Development

Imaging methods potentially provide highly cost-effective, general approaches for the noninvasive characterization of disease and pharmacokinetics, pharmacodynamics and drug effects directly in humans. PET allows the distribution of radiolabeled molecules to be mapped, enabling studies of molecule distribution and tissue metabolism. MRI was initially used primarily to define tissue structure, but a more recent range of functional MRI techniques promise the potential to define pharmacokinetic data over biologically meaningful timescales. With an understanding of the relationship between imaging biomarker changes and clinical outcomes, imaging can be used as a surrogate marker of response even for the later stages of drug development. Two common pitfalls in the application of these methods, which need to be avoided, are:

- Getting distracted by the technology rather than focusing on the questions that need to be asked.
- Failure to interpret the imaging data in an appropriate disease- and drug-specific manner.

However, with attention to such issues, imaging should prove a powerful facilitating platform for experimental medicine in the future. Molecular imaging techniques are already being used in receptor occupancy studies and with transgenic animal models to validate drug development. In clinical trials, molecular imaging probes will also play an increasingly important role in developing new, smarter, and safer drugs for patients. The appropriate use of molecular imaging in drug discovery and development could significantly speed up the development process and save millions of health care dollars.

Biomarkers in Clinical Trials

Interest is increasing rapidly in the use of surrogate biomarkers as primary measures of the effectiveness of investigational drugs in definitive drug trials. Many such surrogate markers have been proposed as potential candidates for use in definitive

effectiveness trials of agents to treat neurologic or psychiatric disease, but as of now, there are no such markers that have been adequately "validated," that is, shown to predict the effect of the treatment on the clinical outcome of interest. There is need for discovery of novel pharmacodynamic biomarkers for use in optimal dose selection in phase II clinical studies. Novartis is using biomarkers in phase IV trials, especially in oncology, CNS, and cardiovascular. The opportunity in these trials is to study the disease, gain samples for additional testing, and define subsets of patients (for patient stratification based on risk or efficacy). The regulatory aspects of biomarkers and validation are discussed in Chap. 19.

Many diseases with long onset and long time to progression require clinical trials that take years and large numbers of patients to determine the benefit of biomarkers or new treatments. For such indications, establishing the value of biomarkers will be longer term. However, biomarkers might have a positive impact on metabolic diseases, such as diabetes much sooner. Diabetes clinical trials using biomarkers can show effects in patients on a timeline that is shorter than using traditional clinical measurements. On the other hand, immunology and asthma are considered two therapeutic areas in which biomarkers are not expected to have an impact in the near future.

NIH Recommendations on the Use of Biomarkers in Clinical Trials

NIH's strategies for improving clinical trials and the recommendations relevant to biomarkers are:

Validate biomarkers and surrogate end points before basing policy guidelines for public health on them The shift in patterns of disease from acute infections to the chronic degenerative diseases affecting older populations has expanded the market for drugs. It has also created a growth industry in biomarkers, since the latter have the potential to allow for smaller trials of shortened duration.

An example is the prostate-specific antigen (PSA) as a biomarker. Tests showing high serum PSA values in men with prostate cancer drove clinical decisions for biopsy and subsequent medical or surgical treatment. The Prostate Cancer Prevention Trial was a randomized placebo-controlled clinical trial enrolled men with PSA levels of 3 ng/ml or under. The men in the trial were treated with the 5-alpha reductase inhibitor finasteride, which lowers testosterone levels, or a placebo, and treatment was followed with periodic PSA tests and digital rectal exams for 7 years. All were offered biopsy at the trial's end. The prevalence of prostate cancer in the finasteride group was 18.4%, compared with 24.4% in the placebo group. However, more than half of the men found with cancer in the placebo group had had a normal PSA and digital rectal examination throughout the trial. In a follow-up study of men in the placebo group, investigators concluded that there is no cutpoint of PSA such that higher scores are strongly associated with higher risk for clinically important prostate cancer and lower scores with lower risk; there are too many false positives and false negatives at every score. The PSA test can detect a broad spectrum of

prostate cancer but it is a heterogeneous group, which ranges from rapidly progressing and aggressive to slowly progressive or nonprogressive forms. Treating the last group may lead to the erroneous conclusion that these patients were "cured" by screening and treatment. If unscreened and untreated, these same men might simply have gone on to die from other causes at the same point in time. Early diagnosis based on screening may also lead to the erroneous assumption that screening increases true survival time, since survival is measured from the time of diagnosis – longer in the case of the screening diagnosis compared with the time when the patient becomes symptomatic (the "lead time" bias).

Caution on the use of surrogate (intermediate) end points Biomarkers can also serve as "surrogate" or "intermediate" end points, instead of true health or clinical. The virtue of a surrogate end point is that it provides a window at an intermediate point in the trial, short of the true health or clinical outcome, and can serve as a bellwether that indicates whether treatment x is working or not, thus saving both time and money. But that depends on the validity of the surrogate. And validity is not easy to establish, as shown by the Diabetes Control and Complications Trial, a randomized trial of patients with diabetes to determine if intense monitoring of glucose and the use of an insulin pump would reduce the risk of retinopathy. The development of microaneurysms was chosen as a surrogate marker, since they are associated with vision loss. Early trends indicated an increase in microaneurysms and could have led to premature termination of the trial. But longer-term follow-up showed definite reduction in visual impairment, and the trial was appropriately ended at that point since it demonstrated a health benefit.

Recognizing their potential value, several methods for the validation of biomarkers and surrogates were proposed at the meeting. One entails the use of hazard rates. The hazard rate is the risk of an event (such as death) at a given point in time in a clinical trial and can be computed for the experimental and control groups in the trial. The hazard rate for the experimental group divided by the hazard rate for the controls defines the "hazard ratio." If this fraction is greater than one, the chances of succumbing to the health risk (such as death) increase with the treatment; if the ratio is less than one, the chances of the health risk decrease with treatment. The hazard rate framework could be used to establish, at the strongest, a causal link between a surrogate and the true clinical or health end point (essentially establishing that the surrogate captures or mediates the relationship between the treatment and the true end point), or less stringently, a strong association. Also strengthening the case for validity would be corroborative findings from meta-analyses of smaller trials of a surrogate in relation to a given therapy. Other tests of validity for biomarkers invoke statistical measurements indicating that the marker demonstrates high sensitivity and specificity or has high predictive value, in an independent test sample. To validate surrogate end points, a "validity" trial in which both surrogate and true end points are observed should be conducted, one in which it can be concluded that the inferences about the intervention were the same, whether based only on the surrogate or only on the true end point.

Table 4.1 Causes of failures in clinical trials and their reduction by use of biomarkers

Causes of failure	Use of biomarkers to reduce failure
Drug given to non-responders in a mixed population of responders and non-responders	Use of biomarkers to select optimal responders to the drug for inclusion in clinical trials
Inappropriate dose as blood levels of the drug do not correlate with adequate occupancy of drug receptors	Use of pharmacodynamic biomarkers to determine optimal dose based on receptor occupancy determined by ligand displacement PET
Clinical efficacy measures may not be objectively measurable or may occur too late during a chronic disease	Use of efficacy biomarkers that enable early and quick assessment of the ability of the drug to alter the pathophysiology of the disease
Toxicity	Use of toxicity biomarkers to determine presymptomatic toxicity

Advantages of Biomarkers for Drug Development

The major expense of drug development is on clinical trials, which account for approximately 60% of the total drug development costs. Failed clinical trials raise the average cost of drug development. Using biomarker-based tests for drug safety and efficacy as well as for stratifying the patients could significantly reduce the duration and cost of clinical trials.

More than 50% of failures in drug development occur in phase II clinical trials. Therefore, reduction of risk of failure prior to phase II would reduce the cost of drug development. Table 4.1 shows some causes of failures in clinical trials and their reduction by use of biomarkers.

Limitations and Problems with Use of Biomarkers in Clinical Trials

High expectations of value of biomarkers to study the pharmacodynamics and pharmacokinetics of drug candidates sometimes lead to disappointments. An assay developed to identify a biomarker may fail due to several reasons. There may be poor communication between the laboratory and the clinical team and the assay vendor may have calibrated them differently from the pharmaceutical company's protocol. Another problem may be poor collection of the blood sample for assay. Those who use the CRP biomarker to track disease progress or drug efficacy may not take into account the fact that CRP levels in some individuals fluctuate throughout the day and during seasonal changes. An assay that works in a phase I study may not be applicable in a phase II trial. The earlier trial is done on a smaller number of healthy people not on medication, whereas patients in the larger phase II trials are likely to be taking medication that can interfere with the assay results.

A biomarker may not correlate well with traditional clinical endpoints if it more sensitive than the traditional endpoints. In pathway-based therapeutic approaches, biomarkers need to be correlated with the characteristics of the pathways. A biomarker may fail if:

- It is not in the disease pathophysiological pathway.
- It is not in the pathway affected by the therapeutic intervention
- The therapy acts through different and unknown pathways.

Application of Biomarkers by the Pharmaceutical Companies

Almost all major pharmaceutical companies are using biomarkers, at least in preclinical drug development. Many of these have in house biomarker programs, whereas others use services of many biotechnology companies involved in supporting biomarker drug discovery and development. These companies generally pursue biomarkers in relation to pathway analysis and in conjunction with hypothesis-driven, deductive, knowledge-based target discovery. However, work on biomarkers presents a scientific opportunity to rediscover biology by better understanding individual diseases and their associated pathways. Currently biochemical assays and imaging are used by most of the companies but the use of metabolomic markers is expected to increase in the future as a part of the increasing use of "omic" technologies. Expression profiling is also increasing in importance. Although technologies for biomarkers have been adopted widely, the maximum utility of these has not been achieved as yet. Biomarker-based drug development some of the major pharmaceutical companies is shown in Table 4.2.

Role of Biomarkers in Vaccine Development

Most traditional vaccines are based on inactivated or attenuated pathogens or on purified pathogen subunits, such as toxins or polysaccharides. These vaccines are quite efficient in preventing infections of pathogens with a low degree of antigen variability because they work by eliciting functional antibodies that can (i) counteract viral invasion, (ii) neutralize bacterial toxins and (iii) induce complement-mediated killing of bacteria (Germain 2010). Vaccines containing multiple antigens (multivalent) have also been produced to cope with the viruses and bacteria that are capable of more moderate degrees of antigen variability (Pomfret et al. 2011). Strategies that have been employed to manage bacterial and viral diversity are based on new technologies that can be applied to the vaccines to prevent infection by highly variable pathogens. These include the use of reverse vaccinology, and analytical and structural vaccinology to cope with either bacterial or viral diversity.

Table 4.2 Biomarker-based drug development at major pharmaceutical companies

Company	Approaches	Examples
Novartis	Multiple approaches, i.e. more than one type of biomarker (DNA, RNA, protein, biochemical) is considered be essential to detection. Novartis has early biomarker plans in place for nearly each compound.	Novartis evaluated genotypes and identified 12 SNPs in six genes that permitted researchers to stratify patients in a CNS clinical trial to maximize the difference in response after 4 and 12 weeks of treatment.
Eli Lilly & Co	Many different markers are pursued simultaneously, including DNA, RNA, protein, and metabolites; the goal is to think in terms of systems biology when deciphering the data. 80–90% of clinical trials phases I- II have some form of biomarker measurement to support the elucidation of drug activity, safety, and efficacy parameters.	Novel biomarkers, not previously associated with sepsis, were used in clinical trials of Xigris (drotrecogin), a new drug for sepsis.
Bristol-Myers Squibb	A mix of technologies chosen to create a panel depends on the biological endpoint being investigated, particularly biologically heterogeneous endpoints. Biomarkers are used in phase II and III trials of competitive compounds in the same class. Class-specific rather than drug-specific biomarkers are considered to be more cost effective at the physician/patient level, which is an important consideration in promoting the widespread use of personalized treatment.	Ixabepilone (BMS 247550) and Taxol (paclitaxel) both affect polymerization of tubulin, preventing cellular division. To see if they share some markers for drug response, tests were done in human cell lines and animals and showed that gene changes from IC50 response in a human cell line predictive model had similarities with a response profile in animals. By the time the compound progressed to an early phase II trial, the markers that had been identified were used in human patients to predict drug response of the tumor.
Bayer AG	Biomarkers are included in nearly drug discovery and development all programs at some level.	Since oncology is a top priority, nearly all clinical trials in this area include biomarkers
Pfizer	80–90% of clinical trials phases I- II have some form of biomarker measurement. The exceptions are situations when efficacy trials are simple and inexpensive to conduct, or when traditional tests are so good that biomarkers are not expected to substantially improve decision making.	
Roche	The search for predictive biomarkers should begin during preclinical research. Ideally, predictive marker candidates should have been identified by the time early clinical development starts.	Predictive markers can help to focus clinical development and lead to more efficient drug development. Co-development of diagnostic test is critical.
Merck & Co	Merck is using an advanced, integrated biomarker approach to R&D to help eliminate failures sooner and bring innovative products to patients faster. Using an integrative genomics approach to elucidate complex traits like disease and drug response, Merck scientists have identified and validated several novel drug targets and biomarkers.	Merck uses phosphoproteomics to identify potential biomarkers for predicting the efficacy of drugs targeting a cancer-linked signaling pathway.

Novel adjuvants, delivery systems, new viral vectors and prime-boost strategies can be used to elicit multifunctional adaptive responses during the vaccination protocol. Systems vaccinology approach is used to better understand correlates of vaccine efficacy and evaluate quality of humoral and cellular responses to vaccination (Michán et al. 2012). Biomarkers of immune response will play an important role in vaccines of future.

A major challenge for vaccinology is that there is no way to measure germinal center activity, particularly in human clinical trials of candidate vaccines (and most nonhuman primate studies of candidate vaccines), because germinal centers (GCs) are the engines of antibody (AB) affinity maturation, which is the goal of all AB-eliciting vaccines. For example, significantly higher levels of plasma CXCL13 (chemokine C-X-C motif ligand 13) are associated with the generation of broadly neutralizing ABs (bnABs) against HIV in HIV-infected individuals. GCs optimize B cell AB responses and are required for almost all B cell receptor affinity maturation; thus providing a critical parameter to monitor if HIV bnABs are to be induced by vaccination. However, lymphoid tissue is rarely available from immunized humans, making the monitoring of GC activity by direct assessment of GC B cells and germinal center CD4+ T follicular helper (GC Tfh) cells problematic. The CXCL13–CXCR5 (chemokine C-X-C motif receptor 5) chemokine axis plays a central role in organizing both B cell follicles and GCs and is a biomarker of germinal center activity (Havenar-Daughton et al. 2016). The authors show explicit relationships between plasma CXCL13 concentrations and germinal center frequencies in lymph nodes in a series of different conditions, including licensed and experimental vaccines, and in humans, nonhuman primates, and mice. These findings support the potential use of CXCL13 as a plasma biomarker of GC activity in human vaccine trials and other clinical settings.

Role of Biomarkers in Relation to Stage of Drug Discovery and Development

The stage for biomarker discovery varies across the pharmaceutical industry, but the trend is definitely toward conducting it early in the drug development phases. The time a compound is identified as a serious candidate for advancement into the clinic is when biomarker discovery begins. Once a candidate biomarker is identified, an assay is developed and the biomarker is extensively tested and validated both at the preclinical stage using animal models and in patients during clinical trials.

Validation in patients is certainly most crucial because it helps establish the association of the biomarker with a certain disease and its severity. It is also the most difficult part because validation involves linking together a number of different studies. Biomarker validation is easier when working with are biologicals such as proteins or antibodies because cycle times are much shorter and there is continuous feedback between discovery and development. Role of biomarkers in drug development in therapeutic areas of cardiovascular and neurological disorders will be discussed briefly in the following sections and drug development in oncology will be discussed in Chap. 13.

Role of Biomarkers for Drug Development in Cardiovascular Disorders

The cardiovascular therapeutic area is complex and includes a number of overlapping diseases. In the past, low-cost biomarkers, such as blood pressure and cholesterol measurements were used. However, they do not address issues such as plaque stability and size. Many new biomarkers have been discovered in recent years (see Chap. 15). Many of these are bases for diagnostic tests and there have potential uses in drug discovery and development. There is need for better diagnostic tests including those encompassing metabolic syndrome – a constellation of disorders including cardiovascular diseases, diabetes, and obesity. Other clinical biomarkers for cardiovascular diseases will include intravascular ultrasound, and in vivo tests for plaque composition and stability using imaging. Biomarkers will be important for development of personalized therapies for cardiovascular disorders.

Role of Biomarkers for Drug Development in Neurological Disorders

The ideal biomarker for CNS drug development should recognize the mechanism of action of a potential new therapy (mechanism-based biomarker) and the relation between biomarker endpoint and intervention should have a biologically plausible explanation. Biomarker endpoints need to be investigated in both animals and humans, as the extrapolation of animal models of disease to human pathology is often uncertain. Validation process is required for a better definition of the biomarker sensitivity, specificity, positive and negative predictive value, accuracy, likelihood ratio of positive and negative tests, discriminant validity, sensitivity to change and to treatment difference.

Improvements in biomarker-based drug discovery in neurological disorders will take place first in diseases without satisfactory treatment, e.g. neurodegenerative disorders, schizophrenia, and pain. The biology of these diseases is not well understood, and the phenotype is complex. This hampers the biomarker discovery and development process, but there is promise in case of Alzheimer's disease to assess the benefit of treatment using imaging and other biomarker techniques (see Chap. 14). Complex diseases, such as schizophrenia and depression, to be most difficult, with biomarker applications developing at a later date.

Neuroimaging is a key biomarker that provides a unique bridge from the laboratory to the clinic in CNS drug development as quantitative biomarkers as surrogate efficacy measures are often lacking and clinical trial endpoints can be confounded by high placebo response. It can be used preclinically to select candidate drug molecules during drug discovery and clinically to facilitate proof of concept testing and optimization of resources through prioritization of decision making during the development of new therapeutics.

Significance of Biomarkers in Drug Development

Understanding the molecular basis of disease will limit failure in drug development due to wrong biological hypotheses. Most of the emphasis on biomarkers in drug development is on early identification of potentially successful molecules to predict potential efficacy and safety. However, it is important that biomarkers be chosen, developed and evaluated in a way that enables them to provide confidence to terminate a molecule when it has no effect on the biomarker. This is clearly a challenge for novel molecules that are the first in their class, but it can be achieved through building a sound theoretical rationale for the biomarker supported by evidence of linkage to the effect of the drug in appropriate animal models. It is worthwhile to develop biomarkers for exploring the pharmacology of new molecules as well as to develop potential biomarkers of efficacy. A molecule that does not have the intended pharmacological effect is unlikely to have the desired efficacy and its development should be terminated. Discovery efforts would then be directed at understanding the reasons for the lack of pharmacological effect and finding improved molecules. For those molecules that have the intended pharmacological effect but then fail to show efficacy, it is possible to say with confidence that the molecular target is ineffective and that discovery effort should be directed to other targets. Such an approach will increase the overall success rates of the candidate molecules delivered into clinical development.

Pharmacogenomic Biomarker Information in Drug Labels

A review of 1200 drug labels of FDA-approved drugs in the US from 1945 to 2005 revealed that 121 contained pharmacogenomic information: 69 referred to human genomic biomarkers, and 52 referred to microbial genomic biomarkers. Of the labels referring to human biomarkers, 43 (62%) pertained to polymorphisms in cytochrome P450 enzyme metabolism, with CYP2D6 being most common. Of 36.1 million patients whose prescriptions were processed by a large pharmacy benefits manager in 2006, about 8.8 million, i.e. approximately one fourth, received one or more drugs with human genomic biomarker information in the drug label (Frueh et al. 2008). The study concluded that incorporation and appropriate use of pharmacogenomic information in drug labels should be tested for its ability to improve drug use and safety in the US. The number of drugs with pharmacogenomic information in labels is increasing.

Organizations and Resources for Biomarker-Based Drug Development

For larger projects, participation in multipartner consortia remains attractive because it brings together participants whose combined expertise is essential for achieving the objectives. Partners in such consortia include pharma, biotech, and diagnostics

companies, as well as universities, governmental agencies, device manufacturers and regulators. Project objectives could include the discovery of disease-related biomarkers using various technological approaches or qualification of candidate biomarkers in larger patient cohorts to enable clinical disease management or progression to surrogate regulatory endpoints in clinical trials. Some funding programs such as those by the European Commission encourage research grant applications from such consortia. The synergistic collaboration between pharmaceutical and diagnostic industry would provide a mutually attractive model whereby pharma's investment in biomarker discovery could be returned through the licensing of diagnostic intellectual property.

Biomarker Alliance

MDS Pharma Services, a provider of innovative drug discovery and development solutions, has formed The Biomarker Alliance (http://www.biomarkeralliance.com/) serving the pharmaceutical and biotechnology industries. The Biomarker Alliance will design and execute a wide range of biomarker discovery and development programs. It is made up of biomarker service providers:

1. Caprion Pharmaceuticals: for protein biomarker discovery.
2. Gentris Corporation: provider of applied clinical pharmacogenomic services.
3. Massachusetts General Hospital Department of Radiology: imaging biomarkers and the use of imaging technology in clinical trials.
4. MDS Pharma Services: provider of biomarker discovery, development and implementation.

The Biomarker Alliance is the only organization providing single-point access to proteomics, pharmacogenomics, imaging, assay development and clinical testing together in one, easy-to-access package designed to maximize the probability of success of any drug candidate. The members of the Alliance have an idea of what it will take to identify and apply biomarkers in a scientific manner that expresses the safety and efficacy of new compounds that could potentially change the way medicine is practiced today.

More research is emerging that shows biomarkers are the wave of the future with the potential to change medicine. The Biomarker Alliance is on the crest of that wave with its collective insight, expertise and seamless program management. These capabilities enable the Biomarker Alliance to significantly reduce drug discovery and development timelines while eliminating the need to invest in multiple technology platforms and infrastructure.

Biomarkers Consortium

The Biomarkers Consortium (http://www.biomarkersconsortium.org/) is a public-private endeavor aimed at developing, discovering, and qualifying new biomarkers for drug development, preventive medicine, and medical diagnostics. It was founded by the NIH, the FDA, the Centers for Medicare and Medicaid Services, and the Pharmaceutical Research and Manufacturers of America in 2006. It now has approximately 30 partners, including companies, patient advocacy organizations, government groups, and non-profit organizations.

The policies and procedures developed by the Biomarkers Consortium are intended to outline general principles to facilitate the use of data and technologies in expanded biomarker research and development efforts conducted by the Consortium while ensuring compliance with relevant requirements of antitrust and other federal laws.

In 2008, the Consortium announced its strategic focus on addressing High-Impact Biomarker Opportunities. These High-Impact Biomarker Opportunities represent pragmatic, high-priority areas of opportunity for biomarker identification, development, and qualification that promise to expedite therapeutic development and improve patient diagnosis, care, and treatment. Key criteria for defining High-Impact Biomarker Opportunities are:

- Important: addresses a significant unmet medical or scientific need in biomarkers with a potentially considerable impact on public health.
- Translational: will result in significant improvement in the development, approval, or delivery of care to patients (i.e. diagnostics, therapeutics, clinical practice).
- Transformational: addresses critical gaps in the biomarkers qualification/validation process and/or may otherwise transform the process of how biomarkers are developed, approved, and applied in the future.
- Feasible: an idea or program whose end goals can likely be achieved in a specific timeframe, and that has a reasonable prospect of producing the expected outcomes. Ideal programs are those which could result in regulatory qualification of a biomarker in 3 years.
- Practical: leverages preexisting resources (e.g., intellectual capital, personnel, facilities, specimens, reagents, data) wherever possible.
- Fundable: is capable of generating the required funding and stakeholder support needed for implementation.
- Collaborative: would uniquely benefit from the multi-stakeholder composition and approach of The Biomarkers Consortium, and could be feasibly executed under its policies.

The consortium provides the best possible mechanism for biomarker development and standardization because it provides common ground on which both the public and private sectors can integrate and leverage their scientific, intellectual, and

financial resources and insights. Project concepts approved by the Consortium are developed into detailed, comprehensive project plans which are approved by its leadership before the Foundation for NIH undertakes formal fundraising and projects are implemented. Projects launched by the Consortium to date include:

- FDG-PET as a biomarker for clinical trials of non-Hodgkin's lymphoma and NSCLC.
- Adiponectin as a biomarker of glycemic control in patients with type 2 diabetes.
- Carotid MRI as a biomarker in AIM-HIGH (Atherothrombosis Intervention in Metabolic Syndrome with Low HDL-cholesterol/High Triglyceride and Impact on Global Health Outcomes) Substudy, to improve patient management by validating a tool that can help identify therapeutic response and facilitate drug development.

Molecular Libraries and Imaging Roadmap of NIH

The Molecular Libraries and Imaging Roadmap initiative of NIH is relevant to biomarker-based drug development. The Roadmap offers public sector biomedical researchers access to the large-scale screening capacity necessary to identify small molecules that can be optimized as chemical probes to study the functions of genes, cells, and biochemical pathways. This will lead to new ways to explore the functions of genes and signaling pathways in health and disease. NIH anticipates that these projects will also facilitate the development of new drugs, by providing early stage chemical compounds that will enable researchers in the public and private sectors to validate new drug targets, which could then move into the drug-development pipeline. This is particularly true for rare diseases, which may not be attractive for development by the private sector.

The initiative will also enhance the discovery and availability of small molecules for molecular imaging. This includes imaging of molecules or molecular events in biological systems that span the scale from single cells to whole organisms. Ultimately, it is hoped that this effort will enable personalized profiles of cell and tissue function, which may lead to more individualized approaches to diagnosing and treating disease. By significantly enhancing the support of this emerging field, NIH will ensure that molecular imaging will become a powerful tool for biomedical research and will be a synergistic component of research in molecular medicine that promises landmark improvements in clinical care.

This initiative will be important for the discovery of biomarkers and their use in drug development. Further details of the NIH Roadmap can be seen at the web site: nihroadmap.nih.gov.

Rare Diseases Clinical Research Consortia

Rare diseases are defined as conditions that afflict <200,000 persons, but there are an estimated 7000 of these disorders and they affect ~25 million persons in the US. In August 2013, NIH announced that its plan to provide ~$17.5 million in funding in 2014 to support a network of as many as 14 Rare Diseases Clinical Research Consortia (RDCRC) that will pursue clinical research projects focused on rare diseases. Each consortium will focus on at least three rare diseases, which should be related, and may be defined on the basis of genetic, genomic, or acquired differences, pathogenesis, or molecular, biochemical, cellular, and other features. For example, these consortia may seek to investigate disease-causing variants in the same gene that lead to different phenotypes or variants in diverse genes that lead to overlapping phenotypes. Clinical data management, including data collection, mining, and sharing, will be addressed through the Data Management and Coordinating Center component of the RDCRN. The consortia will engage in collaborative research projects, train new investigators, conduct pilot and proof-of-concept clinical projects, and share information about these diseases. Each of these consortia will involve multiple institutions and partnering organizations, such as patient advocacy groups. A major aim for these centers will be to investigate potential biomarkers for disease risk and severity and to measure clinical outcomes that could be applicable to clinical trials.

Future of Biomarker-Based Drug Development

Currently <50% of pharmaceutical R&D is improved by postgenomic biomarkers but this is expected to increase to >80% by 2020. The percentage varies considerably according to individual diseases. The number of clinical trials using biomarkers is increasing and most of clinical trials at major pharmaceutical companies will have biomarkers included in the protocol by 2020. In some cases biomarkers will used mainly to identify responders to treatment prior to enrollment. In other cases, a biomarker strategy will be needed to gain the management's approval for compounds to advance. The use of biomarkers would become widespread for clinical use in oncology including use as companion diagnostics for therapeutics. There will be an increase in the use of panels consisting of multiple biomarkers, e.g., DNA plus protein, RNA plus protein, and it will be facilitated by new instrumentation that allows two types of molecules to be detected simultaneously.

An independent development by the biotechnology companies of biomarker-based diagnostic tests to predict response to drugs is to be expected. Such a development may be sponsored by organizations other than the pharmaceutical industry, e.g., payers who wish to control/rationalize expenditures.

All these developments will facilitate the implementation of personalized medicine that would require collaboration of patients, physicians, diagnostic and pharmaceutical companies, academic institutions, payers, and regulatory authorities (see Chap. 18). The Biomarker Alliance will play an important role in this development.

The following recommendations have been made for future (van Gool et al. 2010):

- A more stringent application of biomarker read-outs in early clinical trials to prove target engagement and mechanistic drug effects to better assess therapeutic potential and safety of new compounds.
- Development of clinically relevant models for different diseases, which are based on biomarker-based pathomechanism, and can more reliably predict the efficacy of a new drug in the indication of interest.
- Increasing interactions between preclinical pharmaceutical scientists involved at the early drug discovery phase and their clinical and translational medicine counterparts.
- Validation and development of translational models and clinically applicable biomarkers through substantial concerted effort of multidisciplinary consortia. This will benefit greatly from the availability of well-annotated clinical samples, through shared comprehensive clinical biorepositories.
- Further development of patient stratification biomarkers. This will be further facilitated by the anticipated availability of individual genome sequencing in clinical trials.

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Chapter 5
Role of Biomarkers in Healthcare

Introduction

Study of biomarkers of various diseases will help to improve the management in the following ways:

1. Providing a better understanding of the disease pathomechanisms.
2. Screening to detect early-stage disease in the asymptomatic population and consider pre-emptive treatment.
3. Establishing definite diagnosis and precise description of the disease
4. Classification of patients by disease subset
5. Improving the determination of prognosis.
6. Providing a basis for development of therapeutics and monitoring the effect of therapeutics on the disease.
7. Identification of patients with a high probability of adverse effects of a treatment
8. Posttreatment monitoring for early detection of recurrence and advancing disease or complications.
9. Predict response to particular therapies and choose the drug that is mostly likely to yield a favorable response in a given patient, i.e. personalized medicine.
10. Combination of diagnostics with therapeutics based on the same biomarker in some cases.

Biomarkers that will be useful for either disease prediction or treatment should have one or more of several properties, including:

- Specific and selective association with illness in a population
- Heritability
- Independently indicate the presence of the disease regardless of the presence or absence of the clinical phenotype
- Co-segregation with disease within families
- Presence in relatives of affected individuals at a higher rate than in the general population.

K.K. Jain, *The Handbook of Biomarkers*, DOI 10.1007/978-1-4939-7431-3_5

A biomarker should fulfill three criteria to be useful clinically:

1. Accurate, repeated measurements must be available to the clinician at a reasonable cost and with short turnaround times.
2. The biomarker must provide information that is not already available from a careful clinical assessment.
3. The measured level should aid in medical decision making.

Although one single biomarker fulfils all these conditions, multiple relevant disease biomarkers that can be examined concurrently increase diagnostic specificity. Important therapeutic areas that are currently the focus of biomarker discovery are cancer, metabolic disorders, inflammatory disorders and diseases of nervous system and cardiovascular system. Some diseases overlap within these categories. Many diseases have multiple biomarkers due to involvement of different pathways. Some biomarkers, e.g. those of inflammation occur in several diseases characterized by inflammation.

Biomarkers of Inflammation

Inflammation is an important part of normal responses to infection and injury. However, chronic activation of the immune system, due to aberrant responses to normal stimuli, can lead to the establishment of a persistent inflammatory state, which is a component of many disorders that are discussed according to the system involved. Major disorders with inflammation involve cardiovascular and nervous systems. Diseases such as diabetes and rheumatoid arthritis have are characterized by inflammation. The complement system is activated in virtually all inflammatory diseases and should therefore serve as a fertile source of biomarkers of inflammation. Traditionally, complement activation has been monitored by measurement of serum C3 and C4, the parent molecules and substrates for enzymatic activation. However, these assays are known to have limited utility for monitoring the inflammatory process.

ESR and CRP as Biomarkers of Inflammation

Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are two biomarkers of inflammation that have been commonly used in medical practice. There is strong (but incomplete) correlation between ESR and CRP values. CRP is a direct and quantitative measure of the acute phase reaction, an indicator of inflammatory activity and tissue damage, which correlates closely with the changes in

inflammatory activity due to its fast kinetics. The ESR is an indirect measure of the acute phase reaction and reacts much more slowly than CRP to changes of inflammatory activity. Both ESR and CRP are valuable in discriminating pathology from harmless, often self-limiting diseases. ESR may be useful in the following:

- Establishing a "sickness index" in elderly persons who have nonspecific changes in health status and moderate probability of underlying disease.
- Screening for infection and monitoring treatment response for infections in specific settings.
- Diagnosing and monitoring temporal arteritis, polymyalgia rheumatica, and possibly other rheumatic diseases.
- Monitoring patients with treated Hodgkin disease or prostate cancer.
- Assessing iron deficiency in anemia of chronic disease (when correlated with serum ferritin level).

CRP may be useful in the following situations:

- Differentiation between a bacterial and a viral infection.
- Differentiation between a bacterial infection and an exacerbation of diseases like systemic lupus erythematosus.
- Monitoring of the effect of treatment (with serial measurements)
- Early detection of postoperative complications or intercurrent infections.

Metabolic Biomarkers of Inflammation

Resting nonproliferative tissues have distinctive metabolic activities and requirements, which differ considerably from those in infiltrating immune cells, which are undergoing proliferation and differentiation. Immune responses in tissues may therefore be modulated by the relative abundance of substrates in the inflamed site. In turn immune cell activity can feed back and affect metabolic behavior of the tissues, as most clearly demonstrated in cachexia - the loss of cellular mass driven by TNF-α a key mediator of the inflammatory response. Metabolomic analysis has the potential for to clarify the interactions between inflammation and metabolic changes underlying many diseases. An increased understanding of the interaction between inflammation and cellular metabolism, energy substrate use, tissue breakdown biomarkers, the microbiome and drug metabolites, may provide novel insight into the regulation of inflammatory diseases. Metabolic processes do not occur in isolation, and the interaction of the multiple systems involved in an inflammatory response provides a characteristic fingerprint of disease. (Fitzpatrick and Young 2013). Examples of some metabolic biomarkers of inflammatory diseases that can be detected in body fluids are shown in Table 5.1.

Table 5.1 Metabolic biomarkers of inflammatory diseases

Metabolite/association	Body fluids	Diseases
Lactate/hypoxia	Synovial fluid, urine, CSF	Rheumatoid arthritis Osteoarthritis Multiple sclerosis
Malate/citric acid cycle	Urine	Osteoarthritis
Ketone bodies/amino acid metabolite	Synovial fluid, CSF	Rheumatoid arthritis Multiple sclerosis
Lipoprotein-associated fatty acids/resting energy source	Synovial fluid, blood	Inflammatory arthritis Osteoarthritis
Essential amino acids/protein breakdown	Serum	Osteoarthritis
Creatinine	CSF	Multiple sclerosis
Formate	Urine	Crohn's disease

YKL-40 as a Biomarker Inflammation and Predictor of Mortality

YKL-40 is an inflammatory biomarker associated with disease activity and mortality in patients with diseases characterized by inflammation and tissue remodeling. In a study of the prognostic value of YKL-40 in an unselected patient population, increased blood level of YKL-40 on admission to hospital compared to healthy controls, was a strong predictor of overall mortality, independent of diagnosis (Mygind et al. 2013). YKL-40 could be useful as a biomarker in the acute evaluation of all patients. YKL-40 biomarker of disease severity, prognosis and survival in patients with ischemic heart disease (see Chap. 15). YKL-40 is also a biomarker of poor prognosis in cancer (see Chap. 13).

Biomarkers of Allergic Disorders

There is little activity so far in developing biomarkers in allergic diseases and for vaccination. In 2012, Canada's Centre of Excellence for the Prevention of Organ Failure (PROOF) and Adiga Life Sciences started collaboration to identify proteomic and genomic biomarkers for monitoring the effectiveness of allergy vaccines. Knowledge gained from the project is expected to lead to a better understanding of how such vaccines work, which will then be used to guide development of molecular tests for diagnosing and managing allergic rhinitis. PROOF and Adiga will use patient samples from the trial for a biomarker discovery and validation program that has been established and refined by PROOF. The partners aim to identify protein and genetic biomarkers that respond to vaccine treatment.

Biomarkers of Oxidative Stress

Free radical reactions involving reactive oxygen species (ROS) and reactive nitrogen species (RNS) contribute to the pathogenesis and progression of several human diseases. Some of the stress biomarkers are described with other diseases in this report. There is a real need to develop biomarkers that reflect free radical reactions in vivo.

1,4-dihydroxynonane-mercapturic Acid

4-Hydroxy-2-nonenal (HNE) is a major product of the lipid peroxidation process that is a consequence of free radical reactions. An enzyme immunoassay (EIA) of the major urinary metabolite of HNE, i.e. 1,4-dihydroxynonane-mercapturic acid (DHN-MA) enables direct measurement of DHN-MA in urine with good sensitivity and specificity. Cross-reactivity is very low with 1,4-dihydroxynonene and with different mercapturic acids except with one other HNE urinary metabolite. Good correlation is obtained between EIA and liquid LC/MS quantitation when analyzing urine samples with different oxidative status.

Oxidized Phospholipids

Phospholipids (PLs) are a major class of polar lipids that are abundantly present within cell membranes and the outer shell of lipoprotein particles. Oxidized phospholipids (OxPLs) are generated from (poly)unsaturated diacyl- and alk(en)ylacyl glycerophospholipids under conditions of oxidative stress. OxPLs exert a wide variety of biological effects on diverse cell types in vitro as well as in vivo and are responsible for the pathophysiological actions of oxidized low-density lipoproteins. OxPLs play a role in the development of several chronic diseases and there is growing interest in their potential use as biomarkers of human diseases listed in Table 5.2.

The most sensitive and powerful method for OxPL analysis is mass spectrometry (MS). Development of electrospray ionization MS (ESI-MS) or atmospheric pressure chemical ionization MS (APCI-MS) has enabled sensitive and efficient metabolic profiling of lipids present in a variety of biological samples (tissues and fluids) including atherosclerotic plaque, brain, plasma and cerebrospinal fluid (Philippova et al. 2014). However, large-scale MS analysis of clinical samples remains a challenge due to the complexity and high costs of the technique. The bulk of existing clinical data on OxPL levels in human disease has been obtained using immunological methods. Use of MAbs for detection of various types of OxPLs as well as better characterization of their target oxidation-specific epitopes by mass spectrometry analysis of the spectrum would help to overcome the limitations of current OxPL detection methods.

Table 5.2 Oxidized phospholipids as biomarkers of various diseases

Cardiovascular diseases
Atherosclerosis
Hypercholesterolemia treated with statins
Coronary artery disease
Myocardial infarction
Diabetes and metabolic syndrome
Diabetes with cardiovascular disease
Diabetic nephropathy
Chronic renal disease
Renal insufficiency patients on dialysis
Neurological disorders
Schizophrenia
Bipolar disorder
Neurodegenerative diseases
Chemotherapy-induced neurological disorders
Pulmonary disorders
Lung injury
Cancer
Bilary strictures due to cholangiocarcinoma and pancreatic cancer
Leprosy: disseminated form

Oxidative DNA Damage

Oxidative injury to macromolecules is implicated in a wide range of pathological conditions. Damage is mediated via free radicals that can be created by a range of agents, e.g. xenobiotics, radiation, ischemia-reperfusion injury and normal metabolic activity. These free radicals damage DNA leading to mutation, carcinogenesis or cell death. 8-oxoguanine is formed by free radical damage to DNA and is a sensitive and specific indicator of oxidative DNA damage. Previously, 8-oxoguanine was difficult to detect, requiring the purification of DNA. However, by utilizing a binding protein with high avidity and specificity for 8-oxoguanine, the OxyDNA Test (Biotrin) provides a simple, convenient and sensitive fluorescence method for the detection of oxidized DNA.

Proteins as Biomarkers of Oxidative Stress in Diseases

Proteins are important molecular indicators of oxidative/nitrosative damage. It is not certain whether the presence of oxidatively/nitrosatively modified proteins has a causal role or simply reflects a secondary effect. Only direct identification and

characterization of the modified proteins in a given disease can decipher the potential roles played by ROS/RNS-induced protein modifications. MS-based technologies have contributed significantly towards a better understanding of disease processes. The study of oxidative/nitrosative modifications, investigated by redox proteomics, is contributing to establish a relationship between pathological hallmarks of disease and protein structural and functional abnormalities. MS-based technologies can be used for discovery of diagnostic biomarkers of oxidative/nitrosative stress, enabling early detection of diseases. Identification and characterization of oxidatively/nitrosatively modified proteins in human diseases has now started.

Testing for Oxidative Stress

Bioxytech® assay kits (Precipio Biosciences Inc) simplify the testing of oxidative, antioxidant, nitrosative and inflammatory biomarkers that are considered to play a central role in many human diseases including cancer, diabetes, atherosclerosis, stroke, Alzheimer's, MS and ALS. Oxidative biomarker test kits includes assays for lipid, protein and DNA determinants of oxidative mediated damage.

Reduced glutathione (GSH), a tripeptide (g-glutamylcysteinylglycine) with a free thiol group, is a major antioxidant in human tissues that provides reducing equivalents for the glutathione peroxidase catalyzed reduction of hydrogen peroxide and lipid hydroperoxides to water and the respective alcohol. During this process GSH becomes oxidized glutathione (GSSG). The GSSG is then recycled to GSH by reduction by the reduced form of beta-nicotinamide adenine dinucleotide phosphate (NADPH), catalyzed by glutathione reductase. When mammalian cells are exposed to increased oxidative stress, the ratio of GSH/GSSG will decrease as a consequence of GSSG accumulation. The measure of the GSSG level, or determining the GSH/GSSG ratio, is a useful measure of oxidative stress and to monitor the effectiveness of antioxidant intervention strategies. BIOXYTECH® GSH/GSSG-412 is a commercially available test for this purpose.

Biomarkers of Hypoxia

The term "hypoxia" generally means a reduced supply of oxygen in the living organism. Hypoxia may be either generalized, affecting the whole body, or local, affecting a region of the body. It is difficult to define hypoxia precisely, but it may be described as a state in which aerobic metabolism is reduced by a fall of pO2 within the mitochondria. In this situation, the partial pressure of oxygen, which in dry air is 160 mmHg, drops to about 1 mmHg, by the time it reaches the mitochondria of the cell. Below this value aerobic metabolism is not possible. Hypoxia is not always a pathological condition as variations in arterial oxygen concentrations

can be physiological, e.g., during strenuous physical exercise. In contrast, "anoxia" implies a total lack of oxygen, although the word is sometimes used as a synonym for hypoxia.

Hypoxia occurs in several disorders such as cardiac arrest, respiratory insufficiency, decompression sickness, and carbon monoxide poisoning (see later in this chapter). The most sensitive organ to the effect of hypoxia is the brain. Hypoxic brain damage after cardiac arrest can be estimated by measurements of concentrations of serum S protein, which is an established biomarker of central nervous system injury. It is a reliable biomarker of prediction of survival as well as of outcome.

Tumor hypoxia is associated with resistance to radiotherapy and poor prognosis. It can be visualized using 18F–MISO-PET imaging. Hyperbaric oxygen (HBO) therapy has been used to counteract tumor hypoxia to make tumors more responsive to radiotherapy (Jain 2017). Another example is anti-PD-1 drug sorafenib, the mainstay of treatment of hepatocellular carcinoma (HCC), which leads to tumor hypoxia by upregulation of CXCR4 and thereby abrogates the efficacy of sorafenib. This has provided a rationale for CXCR4 inhibition as adjunct to sorafenib treatment in murine HCC models (Semaan et al. 2017).

Pathophysiology of Hypoxia

Within the cell, 80% of the total oxygen consumption is by mitochondria, and 20% by a variety of other subcellular organs. The biochemical reactions in these locations serve a variety of biosynthetic, biodegradative, and detoxificatory oxidations. Some of the enzymes involved in the synthesis of neurotransmitters have low affinities for oxygen and are impaired by moderate depletions of oxygen. Some of the manifestations of oxygen depletion are related to "transmitter failure" (decreased availability of transmitter), rather than bioenergetic failure. Hypoxia can potentiate injury due to oxidative stress.

Hypoxia Inducible Factor as Biomarker of Hypoxia and Response to Oxygenation

Hypoxia inducible factor (HIF) senses and coordinates cellular responses to hypoxia. HIFs mediate the ability of the cell to cope with decreased oxygen tension. These transcription factors regulate cellular adaptation to hypoxia and protect cells by responding acutely and inducing production of endogenous metabolites and proteins to promptly regulate metabolic pathways. HIF pathway plays an important role in physiological adaptation, cell survival, pH regulation, and adaptation during exercise. HIF-1α, the most well-established member of the HIF family, is a master regulator for the expression of genes involved in the response to hypoxia in most mammalian cells.

HIF-1α plays a role in the pathogenesis of several diseases and can be modulated by HBO therapy (Jain 2017). Interaction of HBO with HIF is variable according to whether the disease is characterized by upregulation or downregulation of HIF-1α.

Identification of Hypoxia Biomarkers from Exhaled Breath

Pilots may experience in-flight hypoxia during high-altitude aviation. There is a need for non-invasive method to monitor for hypoxic risk during flight. This can be achieved through volatile organic compound (VOC) analysis in the exhaled breath VOCs produced during periods of reduced O2 levels. In study, breath samples from the flight mask were collected and analyzed by gas chromatography/mass spectrometry (Harshman et al. 2015). Seven compounds (pentanal, 4-butyrolactone, 2-pentanone, 2-hexanone, 2-cyclopenten-1-one, 3-methylheptane and 2-heptanone) were found to significantly change in response to hypoxic conditions. Additionally, the isoprene, 2-methyl-1,3-butadiene, increases following the overall exposure profile. This study establishes an experimental means for monitoring changes in VOCs in response to hypoxic conditions.

Metabolic Biomarkers of Hypoxia

The following metabolic disturbances have been observed as a result of experimental hypoxia produced in animals:

- Appearance of excess lactate in the blood
- Appearance of 2,3-DPG in the blood of animals exposed to hypoxia of high altitudes
- Higher plasma levels of corticosterone, leading to neo-glucogenesis
- Decrease of long-chain unsaturated fatty acids in the blood sera of rats adapted to hypoxia

Biomarkers of Liver Disease

Over the past decade, there has been a renewed enthusiasm to develop noninvasive serum markers or tests to assess the presence and severity of fibrosis in chronic liver disease. Although a single biomarker or test has lacked the necessary accuracy to predict fibrosis, different combinations of these markers or tests have shown encouraging results. However, inter-laboratory variability and inconsistent results with liver diseases of varying etiologies have made it difficult to assess the reliability of these markers in clinical practice. Current toxicity testing methods often fail to

identify human liver toxicity issues. Therefore, liver toxicity is often detected for the first time when drugs are in phase II of clinical testing after a considerable amount of money has been spent on a drug. Efforts are being made to develop biomarkers for hepatotoxicity in humans for improved preclinical pharmaceutical tests for liver toxicity. Biomarkers of hepatocellular carcinoma are described in Chap. 13.

Breath Biomarkers of Liver Disease

Breath biomarkers have the potential to offer information that is similar to conventional clinical tests. Breath biomarkers can be used for study of liver disease, particularly non-alcoholic fatty liver disease in which breath ethanol, ethane and acetone can be useful biomarkers. Breath ethanol can be associated with hepatic steatosis, and breath acetone can be associated with non-alcoholic steatohepatitis.

Biomarkers of Liver Injury

Alpha glutathione S-transferase (alpha GST) is a uniquely sensitive and specific biomarker of hepatocyte injury. Unlike the aminotransferases, which are predominantly found in the periportal hepatocytes, alpha GST is found in hepatocytes, alpha GST is found in hepatocytes throughout the periportal and centrilobular regions of the liver. This uniform hepatic distribution, together with high intracellular levels and a short half-life (~90 min) means that alpha GST is a more sensitive and specific indicator of hepatocyte injury in situations such as hepatoxicity, transplantation, ischemia-reperfusion injury and hepatitis

Fibrosis and Cirrhosis of Liver

Fibrosis of the liver can be caused by several diseases that can progress through various stages culminating in cirrhosis where it becomes nonreversible. The current ‘gold standard’ for liver cirrhosis detection is an invasive, costly, often painful liver biopsy. Therefore, there is a need for biomarkers that could obviate biopsy in cirrhosis patients. High serum levels of tropomyosin and MFAP-4 were demonstrated in patients with hepatic cirrhosis due to different causes by using a proteomic approach (Mölleken et al. 2009). A quantitative analysis of MFAP-4 serum levels in a large number of patients showed MFAP-4 as a novel candidate biomarker with high diagnostic accuracy for prediction of nondiseased liver versus cirrhosis.

Efforts are made to detect liver fibrosis at an early stage to attempt prevention of progression to cirrhosis. There are several laboratory tests used to assess liver function. Of all the noninvasive laboratory tests available, hyaluronic acid (HA) is now considered to be the most sensitive biomarker of liver fibrosis. Corgenix has

developed several diagnostic products, manual as well as an automated versions, which measure HA. The HA test kit is an ELISA that uses a capture molecule known as HA binding protein.

FibroMax

FibroMax™ (Lab21 Limited) is a combination of five algorithm tests:

- FibroTest™ measures the level of liver fibrosis.
- ActiTest™ measures active liver disease.
- SteatoTest™ measures hepatitic steatosis or 'fatty liver'.
- NashTest™ measures the level of non-alcoholic steatohepatitis.
- AshTest™ is used to monitor liver damage in cases of severe alcoholic steatohepatitis.

FibroMax™ uses a unique combination of serum biomarkers plus age, gender, weight and height data which, when entered into patented algorithms, accurately determines the level of liver disease without the need to undertake an invasive liver biopsy. Globally, over 120,000 HCV) patients are now being clinically managed using FibroTest™– ActiTest™ as an alternative to liver biopsy. In a comparative study on patients with alcoholic liver disease, other biomarkers FibrometerA and Hepascore, did not improve the diagnostic and prognostic values of FibroTest (Naveau et al. 2009).

Hepatic Encephalopathy

Hepatic encephalopathy is defined as a metabolically induced, potentially reversible functional disturbance of the brain that may occur in acute or chronic liver disease (Jain 2017a). It is characterized by disturbances of consciousness and other neuropsychiatric manifestations, which are due to metabolic disturbances associated with liver disease or portosystemic shunts. A substantial amount of clinical and experimental evidence suggests that ammonia toxicity is a major factor in the pathogenesis of hepatic encephalopathy. However, hyperammonemia does not fully explain hepatic encephalopathy because 10% of affected patients have normal blood ammonia levels.

Examination of CSF is not remarkable. There is no "gold standard" for diagnosing hepatic encephalopathy, and there is need for biomarkers. Serum S100b, an astrocyte-specific protein, is a useful biomarker of hepatic encephalopathy in patients with fulminant hepatitis. A pilot study has shown that determination of 3-nitro-tyrosine in serum is a useful biomarker in patients with minimal hepatic encephalopathy (Montoliu et al. 2011).

miRNA Biomarkers of Liver Disease

miRNAs have been studied in serum samples from patients with accidental acetaminophen overdose, hepatitis B infection, liver cirrhosis and type 2 diabetes as well as gender- and age-matched healthy subjects with no evidence of liver disease (Krauskopf et al. 2017). The miRNA signatures were identified using next-generation sequencing that provided analysis for the whole miRNome, including miRNA isoforms. Compared to the healthy subjects, miRNAs showed altered serum levels across the diseased subjects. Although many subjects have elevated alanine aminotransferase suggesting liver impairments, distinct miRNA signatures were identified for different degrees of impairments with minimum overlap. miRNA signatures in human serum are very specific and can differentiate between acetaminophen-induced injury and hepatitis (Vliegenthart et al. 2015). The serum miRNA signature found in HBV patients consists of 25 miRNAs, revealing a simultaneous increase of the 5p and 3p forms for miR-122, miR-125b, miR-194, miR-455 and miR-99a, which has been previously observed in the case of miR-455 in HBV-infected children. Elevated levels of miR-122, miR-194 and miR-125b could be related to immunity and inhibition of HBV.

Bioinformatics analysis of miRNA signatures reveals relevant molecular pathways associated with the mechanisms of toxicity and or pathogenesis of disease. The high proportion of miRNA isoforms present in the respective signatures indicated a new level of complexity in cellular response to stress or disease. This study demonstrates that signatures of circulating miRNAs show specificity for liver injury phenotypes and, once validated, might become useful for diagnosis of organ pathologies as “liquid biopsies”.

Viral Hepatitis B and C

Histologic examination of a liver biopsy specimen is regarded as the reference standard for detecting liver fibrosis. Biopsy can be painful and hazardous, and assessment is subjective and prone to sampling error.

Among the noninvasive alternatives to liver biopsy, several studies have demonstrated the predictive value and superior benefit/risk ratio to biopsy of two combinations of simple serum biomarkers in patients infected with hepatitis B virus (HBV) and hepatitis C virus (HCV). These include FibroTest (FT, BioPredictive) for the quantitative assessment of fibrosis, and ActiTest (BioPredictive) for the quantitative assessment of necroinflammatory activity (HCV-FibroSURE, LabCorp). These tests, which are now available in several countries, can facilitate the screening and management of the most frequent liver diseases. Although FT has been studied extensively, to date there are only few independent studies. In addition to significant inter-laboratory variations, these studies have shown that significant fibrosis could be missed, or conversely significant fibrosis diagnosed in

the absence of minimal or no fibrosis in about 15–20% of patients. Other biomarkers are being investigated.

Biomarkers of Hepatitis C

Collagen IV, a component of basement membranes, is released into the blood during basement membrane turnover. Increased deposit of collagen, associated with increased serum levels of collagen IV and serum IV, may be an early and specific biomarker for active fibrosis, particularly in alcoholic liver disease and hepatitis C. Elevated serum collagen IV levels are also associated with resistance to interferon therapy.

In HCV-infected hemophilia patients, FT can correctly identified clinically advanced or minimal liver disease. Discordance among the various biomarkers of fibrosis is considerate; nevertheless, practical combination of FT, AST-to-platelet ratio index, and Forns may predict stage of fibrosis with accuracy, potentially avoiding liver biopsy in the majority of the patients.

miR-122 has been proposed as a biomarker for hepatitis C. Decreased levels of miR-122 have been linked to a strain of HCV that is resistant to interferon therapy (Sarasin-Filipowicz et al. 2009). Thus, measurement of miR-122 levels in the bloodstream of HCV positive patients can enable more accurate and personalized therapy for their disease.

Biomarkers of Hepatitis B

Hepatitis B viral load is a strong predictor for liver disease progression. Impact of hepatitis B viral and host factors on the progression of chronic HBV infection has been explored using molecular biotechnologies and some of the findings are (Lin and Kao 2016):

- Serum HBsAg level serves as a complementary biomarker to viral load for the prediction of HBV-related adverse outcomes in patients with low viral load, e.g., higher risks of cirrhosis and HCC.
- Hepatitis B core-related antigen (HBcrAg) induces host immune responses, and the reduction of the HBcrAg level as well as the increase of total anti-HBc level are significantly associated with favorable outcomes.
- HBV genotypes are well known viral genetic biomarkers for prediction of disease progression.
- Serum inflammatory biomarkers have been developed to evaluate the HBV-associated host factors, e.g., inflammation with necrosis and fibrosis of the liver.

These findings indicate that patients with chronic HBV infection should be evaluated with relevant viral and host biomarkers to identify those who are at a higher risk of liver disease progression and then receive timely antiviral therapy.

Biomarkers of Pancreatitis

Acute pancreatitis is an acute inflammation of the pancreas. The most common causes being gallstones and alcohol abuse. The condition ranges from mild to life threatening and the severity of the condition may not be obvious at presentation. A key event in the development of severe pancreatitis is the activation of proenzymes in the pancreas, the most important of which is trypsinogen. Trypsinogen is converted into active trypsin, which lyses pancreatic tissue releasing further pancreatic enzymes and leading to tissue necrosis and inflammation. During the activation of trypsinogen a small peptide, trypsinogen activation peptide (TAP) is split from trypsinogen and its presence in body fluids is a sensitive and specific indicator of severe acute pancreatitis. TAP is a better discriminator of severe acute and acute pancreatitis than other laboratory tests, e.g. amylase or lipase. TAP is difficult to measure, requiring sensitive immunoassay techniques, but it is valuable in the following situations:

- Monitoring the effects of different therapies for acute pancreatitis
- Investigating the effects of surgery on the pancreas
- Investigating the toxic effects of drugs on the pancreas, e.g. anti-HIV therapy
- Urinary TAP (uTAP) has the potential to act as a stratification biomarker on admission for differentiating severity of acute pancreatitis (Huang et al. 2013).

In another study, the global metabolites changes in acute pancreatitis were profiled by using gas chromatography-mass spectrometry (GC-MS) of serum specimens (Xiao et al. 2017). 3-hydroxybutyric acid, phosphoric acid, glycerol, citric acid, d-galactose, d-mannose, d-glucose, hexadecanoic acid and serotonin were selected as potential biomarkers for helping in clinical diagnosis of acute pancreatitis and identification of relevant pathways. Results suggest that GC-MS based serum metabolomics for profiling of potential biomarkers can be used for the clinical diagnosis of acute pancreatitis.

Biomarkers of Renal Disease

Several biomarkers have been tested in prospective studies of chronic kidney disease. Biomarkers of nephrotoxicity were discussed in Chap. 4. Although change in serum creatinine is the standard test for the detection of renal injury, its lack of sensitivity has made the early diagnosis of acute kidney injury very difficult. Despite the early promise of biomarkers such as kidney injury molecule-1 (KIM-1) for the early detection and prognosis of kidney disease, the published clinical studies of urine KIM-1 so far are insufficient to support it as an effective diagnostic test in humans (Fontanilla and Han 2011). Because of the heterogeneity of renal disease, more than one biomarker may be required to obtain sufficient sensitivity and specificity for screening acute kidney injury.

C reactive protein (CRP) has consistently emerged as an early biomarker of renal dysfunction. Measurement of CRP is recommended for monitoring the risk of atherosclerotic complications in patients with chronic kidney disease and end-stage renal disease, particularly in those with cardiovascular complications. The usefulness of this measurement for predicting the evolution of chronic kidney disease or for monitoring the response to renoprotective treatment, however, still remains unproven. There is growing interest in homocysteine and asymmetric dimethyl arginine as biomarkers of cardiovascular and renal risk but the usefulness of these biomarkers in clinical practice remains to be proven. Brain natriuretic peptide and troponin T are strongly related to cardiovascular outcomes in end-stage renal disease patients but their value in this population still requires to be properly tested in specifically designed intervention studies.

Biotrin International has tests for several urinary biomarkers of renal disease, which include glutathione S-transferase, renal papillary antigen and collagen IV (see profile and web site of the company for details). Analysis of prostaglandin F2α, nucleic acid damage products, advanced oxidation protein products, and gas components in the exhaled air as oxidative stress biomarkers are also useful for assessment of patients with chronic renal failure.

Biomarkers of Lupus Nephritis

Systemic lupus erythematosus can affect kidneys, which may sometimes lead to end-stage renal disease. Lupus nephritis is divided into six classes and scored according to activity and chronicity indices based on histologic findings. Conventional clinical laboratory measurements such as proteinuria, glomerular filtration rate, anti-dsDNA and complement levels are not sensitive or specific enough for detecting ongoing disease activity in lupus kidneys and early relapse of nephritis. Treatment differs based on the pathologic findings. Renal biopsy is usually the only way to accurately predict class and activity and chronicity indices. There is a need for biomarkers of disease activity in lupus nephritis, which should predict early subclinical flares and serve as a gauge of response to therapy, thus obviating the need for serial renal biopsies with risks of serious complications. Urine and serum are easily available sources of lupus nephritis biomarkers and proteomic approaches have been used (Soliman and Mohan 2016).

A metabolomic approach using ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UPLC-HRMS) has been applied to serum samples from patients with lupus nephritis, idiopathic nephrotic syndrome, and healthy controls (Li et al. 2017). Compared to healthy controls and idiopathic nephrotic syndrome, patients with lupus nephritis had increased serum levels of sorbitol and glycocholic acid metabolites, as well as decreased levels of cortisol, creatinine and L-aspartyl-L-phenylalanine. These metabolic disturbance may be closely associated with inflammation injury, oxidative stress and phospholipid metabolism. A panel of three metabolomics (theophylline, oxidized glutathione and

capric acid) was identified as biomarkers of lupus nephritis with a sensitivity of 87.50% and a specificity of 67.86%. Results of this study indicate that UPLC-HRMS based quantification of circulating metabolites is a useful tool for identification of biomarkers that can differentiate lupus nephritis from idiopathic nephrotic syndrome, and healthy controls.

Urine samples collected from patients immediately before they undergo kidney biopsy can be studied by 2DGE for proteins that are potential biomarkers for lupus nephritis disease and the results can be compared with the findings from the kidney biopsies. Proteins thus identified can be used as biomarkers to indicate the type and severity of renal disease in these patients, as well as the extent of damage to the kidney. An assay based on antibodies against these spots could eliminate the need for renal biopsy, allow frequent evaluation of disease status, and begin specific therapy for patients with lupus nephritis. Further studies are needed to determine whether urine protein analysis could replace the use of biopsies to assess kidney damage in lupus.

Biomarkers of Diabetic Nephropathy

Clinical management and therapeutic intervention at earlier stage of diabetic nephropathy (DN) is of major importance in prevent the progression to end stage renal disease. Currently, the measurement of albumin in the urine is used as a standard non-invasive test for the diagnosis of early DN. This test, however, does not detect kidney disease in some cases. Therefore, efforts have been made to find better diagnostic biomarkers of DN. Proteomics approaches have isolated potential biomarkers of DN. Further investigations have identified several proteins that can be used as diagnostic biomarkers of DN, including urinary immunoglobulin G, transferrin, ceruloplasmin and serum cystatin-C. A summary of the diagnostic biomarkers developed over the last decade, and comments on their impacts in the diagnosis and management of this disease have been published (Ito et al. 2008).

Cystatin C as Biomarker of Glomerular Filtration Rate (GFR)

Cystatin C is a non-glycosylated protein of low molecular weight (13 kDa) in the cystatin superfamily, which is produced at a constant rate in all nucleated cells. Cystatin C belongs to the cysteine proteinase inhibitor group and is associated with several pathological conditions. Imbalance between Cystatin C and cysteine proteinases is associated with diseases such as inflammation, renal failure, cancer, Alzheimer disease, amyotrophic lateral sclerosis, multiple sclerosis and hereditary Cystatin C amyloid angiopathy. Cystatin C is removed from blood plasma by glomerular filtration in the kidneys. It is reabsorbed by the proximal tubular cells and degraded. There is a linear relationship between the reciprocal Cystatin C

concentration in plasma and the glomerular filtration rate (GFR). Cystatin C is suggested to be a better biomarker for GFR than other markers as its serum concentration is not affected by factors such as age, gender and body mass. There is association of Cystatin C levels with the incidence of myocardial infarction and coronary death, presenting a risk factor for secondary cardiovascular events. The DetectX™ Cystatin C Immunoassay Kit (Luminos LLC) is designed to measure Cystatin C levels. This kit uses a native human Cystatin C molecule as a standard.

Estimated GFR and Albuminuria as Biomarkers of Chronic Kidney Disease

Controversy surrounds the use of estimated GFR (eGFR) and albuminuria as biomarkers of chronic kidney disease and for assigning its stages. A meta-analysis was done to assess the independent and combined associations of eGFR and albuminuria with mortality (Chronic Kidney Disease Prognosis Consortium 2010). The Consortium selected 21 studies including a total of 1,234,182 participants from 14 countries for the analysis. Results show that reduction of eGFR (< 60 mL/min) and elevation of albumin-to-creatinine ratio (>1.1 mg/mmol) are independent predictors of mortality risk in the general population during 2.1–11.6 years (average 8 years) of follow-up. High levels of albumin in the urine indicates kidney damage and markedly higher risk of mortality in these persons than those with low levels of albumin in the urine. Similar findings were recorded for cardiovascular mortality and in studies with dipstick measurements. The study provides quantitative data for use of both kidney measures for risk assessment and definition and staging of chronic kidney disease.

Proteomic Biomarkers of Acute Kidney Injury

AKI, previously referred to as acute renal failure, is an important problem in clinical medicine. Despite significant improvements in therapeutics, the mortality and morbidity associated with AKI remain high. The reasons for this include (a) an incomplete understanding of the underlying pathophysiologic mechanisms, and (b) the lack of early biomarkers for AKI, and hence an unacceptable delay in initiating therapy. Application of functional genomics and proteomics to human and animal models of AKI has uncovered several novel genes and proteins that are emerging as biomarkers and novel therapeutic targets. Identification of protein biomarkers in the plasma (NGAL and cystatin C) and urine (NGAL, KIM-1, IL-18, cystatin C, alpha 1-microglobulin, fetuin-A, Gro-alpha, and meprin) hold promise for the investigation of ischemic AKI (Devarajan 2008). It is likely that the AKI panels will be useful for timing the initial insult and assessing the duration of AKI. Based on the differential expression of the biomarkers, it is also likely that the AKI panels will distinguish between the various causes of AKI, and predict clinical outcomes.

Troponin-T as a Biomarker for Predicting End-Stage Renal Disease

A follow-up of the participants with family history of hypertension (whites from Rochester, Minnesota; African Americans from Jackson, Mississippi) in the Genetic Epidemiology Network of Arteriopathy study has shown that cardiac troponin T (cTnT) is a predictor of end-stage renal disease (ESRD) and death beyond traditional risk biomarkers regardless of race or baseline kidney function (Hickson et al. 2015). At 10 years, those with an abnormal cTnT had a high cumulative incidence of death totaling 47% compared to those with a normal cTnT (7.3%). In addition, 10 years after the initial testing, the cumulative incidence of ESRD was 27% among those with an abnormal cTnT, compared to the substantially lower rate found in those with normal cTnT (1.3%).

Further studies may be needed to determine whether cTnT screening in individuals with hypertension or in a subset of hypertensive individuals would help identify those at risk of ESRD and all-cause death. Early intervention and treatment can be key to stopping kidney disease progression and preventable death.

Biomarkers in Pediatrics

There are characteristics of pediatric medicine that make biomarker research especially needed, there are additional challenges as well. A review focuses on the additional considerations needed for applying biomarker research to children, and recommendations for advancing pediatric biomarker research (Savage and Everett 2010).

Pediatric Critical Care

In pediatric critical care, validated biomarkers are essential for guiding drug therapy. A review has presented examples of current biomarker developments in its full breadth, including biochemical substances, physiological measurements and clinical scoring tools, with a focus on the field of circulatory, renal and neurophysiologic failure (Buijs et al. 2012). Within each field the authors consecutively discuss the rationale for the selected biomarkers, studies in critically ill children, biomarker validation stage and biomarker use or potential use in drug studies and clinical drug dosing. This review shows that there is paucity of properly validated biomarkers. Nevertheless, recent developments in, for instance, the field of sepsis, point us toward a future wherein, for critically ill children, drug therapy may be personalized using proteomic profiling instead of a small number of biomarkers, in order to establish a personal and dynamic disease profile.

Biomarkers of Acute Kidney Injury in Children

Acute kidney injury is a common and significant complication among pediatric patients with congenital heart disease, occurring most commonly after cardiopulmonary bypass. Current laboratory methods of diagnosis are not timely enough to guide management decisions, thus spurring interest in discovering new biomarkers of acute injury. Several promising candidates, including NGAL, IL-18 and KIM-1, have been the subject of recent investigation and may facilitate earlier and more accurate diagnosis of renal injury within this cohort. There is little evidence demonstrating that it will be possible to rely upon one particular biomarker as a single agent, and evidence supports that the use of biomarker panels will be most effective. Further clinical validation and broader commercial availability of these novel biomarkers will probably revolutionize the care of pediatric cardiac patients with renal injury (Kwiatkowski et al. 2012).

Biomarkers of Miscellaneous Disorders

Biomarkers of Carbon Monoxide Poisoning

Levels of carboxyhemoglobin do not correlate with symptoms. Carbon monoxide (CO) poisoning is associated with an elevated level of tau and S100B proteins in the serum of patients who had suffered a loss of consciousness. A study has shown that tau protein is a more sensitive biomarker than S100B protein for the earlier stage of neurotoxic effects of CO intoxication (Gawlikowski et al. 2014). Delayed neurological sequelae of CO poisoning are difficult to predict even after successful treatment in the acute stage. A retrospective study has reported that the level of serum S100B protein is a useful biomarker for evaluating patients with acute CO poisoning as well as an independent predictor of the development of delayed neurological sequelae (Park et al. 2012).

Biomarkers of Castleman Disease

Castleman disease (CD) is a rare disease of lymph nodes and related tissues. It is a heterogenous group of lymphoproliferative disorders that are sometimes associated with HIV and human herpesvirus 8 (HHV-8). Although Castleman disease is not cancerous, it may also be associated with malignancies such as Kaposi sarcoma, non-Hodgkin lymphoma, Hodgkin lymphoma, and POEMS syndrome. The disease is nonclonal, with no IgH or TCR gene rearrangements. The causes of idiopathic multicentric Castleman disease (iMCD), one of the three subtypes of CD, are unknown. About 1000 new cases of iMCD are diagnosed each year in the US and

~35% of patients die within 5 years of diagnosis. iMCD patients experience a wide spectrum of symptoms, from mild flu-like symptoms to acute sepsis-like multiple organ system failure due to uncontrolled immune system activation. The Castleman Disease Collaborative Network (CDCN) brought together physicians and researchers to establish diagnostic criteria for iMCD, which include clinical and pathology features, and diseases to exclude (Fajgenbaum et al. 2017). However, the diagnostic process can be time-consuming as there is significant overlap with malignant, autoimmune, and infectious disorders. There is no definitive test or diagnostic biomarkers for iMCD.

In April 2017, CDCN started to work with Janssen Research & Development LLC to identify molecules whose levels are specifically altered in the blood of iMCD patients as biomarkers that can be used to shorten the time required to diagnose iMCD for critically ill patients and as therapeutic targets for treatment of iMCD patients that do not respond to current therapies. To accomplish these goals, the collaborators will measure the level of ~1300 analytes in >260 serum samples collected from ~100 iMCD patients at various time points during active disease.

Siltuximab, an anti-IL-6 MAb developed by Janssen, was approved by the FDA for treatment of iMCD in 2014. However, the lack of defined diagnostic criteria or disease-specific biomarkers can impede timely administration of treatment before organ dysfunction and death. Many questions related to iMCD still exist, particularly for patients that do not respond to siltuximab.

Biomarkers of Erectile Dysfunction

Erectile dysfunction (ED) is a highly prevalent functional disorder and its incidence increases with advancing age. Population based studies estimate the prevalence at nearly 50% of the male population over the age of 45 across all racial and socioeconomic groups. There are multiple risk factors and biomarkers for ED. Currently serum testosterone is considered the most reliable biomarker for establishing the presence of ED due to hypogonadism. ED is more commonly seen in men with various components of the metabolic syndrome and can be considered as a risk marker of the metabolic syndrome and its associated conditions such as diabetes and cardiovascular disease.

Demographic studies have consistently demonstrated that risk factors for ED generally mirror risk factors also predictive of coronary and vascular disease secondary to endothelial dysfunction of the vascular tree. These risk factors include obesity, hyperlipidemia, hypertension, diabetes mellitus, smoking, sedentary lifestyle among others and in clinical studies these co-morbidities are much higher in the ED population than reported in the general population. Various studies support the concept that ED is a marker of cardiovascular disease. Furthermore, endothelial dysfunction is a biomarker of vascular disease. Flow mediated reflex brachial artery dilatation has been described as a diagnostic test for endothelial dysfunction and a high correlation with erectile dysfunction, but it is not commonly done, difficult to

standardize, costly and time consuming therefore is not an acceptable screening test or procedure.

Coronary endothelial dysfunction (CED) precedes atherosclerosis and is associated with cardiovascular events. Both CED and erectile dysfunction (ED) are partly mediated by impairment in the nitric oxide (NO) pathway. Although ED is associated with established coronary atherosclerosis, its relationship with CED is unknown. Association of CED with ED in men with early coronary atherosclerosis as well as the role of the endogenous nitric oxide synthase inhibitor, asymmetric dimethylarginine (ADMA), is an independent biomarker for cardiovascular disease. ADMA plays a role in the systemic manifestations of endothelial dysfunction.

Daily sildenafil for 4 weeks ameliorates endothelial function in patients with ED as assessed by reduction of endothelin-1 levels and other biomarkers of endothelial function – nitric oxide and cyclic guanosine monophosphate (Angelis et al. 2009). The clinical implications of this finding warrant further investigation.

The availability of a simple, cost effective biomarker to identify men at risk would not only allow for earlier treatment but for earlier evaluation and intervention, which could potentially significantly improve cardiovascular health and prevent or postpone potentially serious life threatening events.

Biomarkers of Fever

Fever is not only observed in the course of a bacterial or viral infection, but can be a symptom of, for instance, auto-immune, malignant or thromboembolic disease. Determining the cause of fever in a fast and reliable way is of pivotal importance, as different causes of fever may require different therapies. Neither clinical signs and symptoms, nor traditional biomarkers, such as CRP, leukocytes and ESR have sufficient sensitivity and specificity to guide treatment decisions. Procalcitonin seems to be the most helpful laboratory biomarker for the differentiation of causes of fever, particularly in autoimmune, autoinflammatory and malignant diseases (Limper et al. 2010).

Biomarkers of Heat Stroke

Heat stroke, also termed “sun stroke,” is characterized by hyperpyrexia with core body temperature greater than 40 °C and neurologic dysfunction. Heatstroke is associated with systemic inflammation leading to multiorgan dysfunction. Early detection and management is essential to improve chances of recovery and survival. There are no validated biomarkers for early detection of heat stroke but studies are in progress to identify these.

Heat shock protein Hsp72 concentration is higher in the serum of runners with symptoms of heat illness than in non-ill runners and is a function of the core

temperature attained rather than the rate of heat storage. Measurement of antibodies to Hsp72 may be useful in assessing how individuals are responding to abnormal heat stress within their living and working environment and may be used as biomarkers to evaluate their susceptibility to heat-induced diseases.

High mobility group box-1 protein (HMGB1) has recently been identified as a late mediator of systemic inflammation inducing multiorgan dysfunction. HMGB1 is released into circulation at an early stage of heat stroke with peak levels occurring within 6–13 h post-heat stroke. In a study, plasma HMGB1 levels remained markedly elevated in the following 6 d post-heat stroke when compared with healthy volunteers (Tong et al. 2011). A serum level of 47 ng/mL of HMGB1 at admission indicated lethality with 77.4% sensitivity and 84.2% specificity. Therefore, HMGB1 level at admission is an indicator of the severity of illness and a useful mortality predictor in exertional heatstroke.

Biomarkers of Hyponatremia

Hyponatremia is common and its differential diagnosis, mainly based on the routine clinical assessment of volume status, is often misleading. Mid-regional pro-atrial natriuretic peptide (MR-proANP) is associated with extracellular and cardiac fluid volume. A prospective multicenter observational study on patients admitted to the emergency department with profound hypo-osmolar hyponatremia (Na <125 mmol/L) used standardized diagnostic evaluation of the underlying cause of hyponatremia and carefully evaluated volemic status using clinical criteria (Nigro et al. 2015). MR-proANP levels were compared between patients with hyponatremia of different etiologies and for assessment of volume status. Results showed that MR-proANP levels were higher in patients with hypervolemic hyponatremia compared to patients with hypovolemic or euvolemic hyponatremia. In multivariate analysis, MR-proANP remained an independent predictor of excess extracellular fluid volume after adjustment for congestive heart failure. MR-proANP predicted the syndrome of inappropriate antidiuresis (SIAD) versus hypovolemic and hypervolaemic hyponatremia. It was concluded that MR-proANP is associated with extracellular fluid volume in patients with hyponatremia, and remains an independent predictor of hypervolemia after adjustment for congestive heart failure. MR-proANP may be a biomarker for discrimination between the SIAD and hypovolemic or hypervolemic hyponatremia.

Biomarkers of Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a spectrum of disorders that affect the gastrointestinal tract, the two major entitites being Crohn's disease and ulcerative colitis. IBD is an enduring disease involving mostly young people, with symptoms of bloody diarrhea and abdominal cramps. Several antibodies have been associated with IBD. The two most comprehensively studied are autoantibodies to neutrophils

(atypical perinuclear anti-neutrophil cytoplasmic antibodies) and anti-*Saccharomyces cerevisiae* antibodies (ASCA). These antibodies are useful for diagnosing IBD, differentiating Crohn disease from ulcerative colitis, indeterminate colitis, monitoring disease, defining clinical phenotypes, predicting response to therapy, and as subclinical biomarkers. Pancreatic antibodies have been described in patients with Crohn's disease. The antigen has not been elucidated and the antibodies are detected by indirect immunofluorescence. There is evidence that the number and magnitude of immune responses to different microbial antigenss (outer membrane porin C and ASCA) in a given patient are associated with the severity of the disease course, i.e. the greater the number of responses and greater their magnitude, the more severe the disease course. The interest in antimicrobial and antiglycan antibodies has recently been increased as they have shown to act as surrogate markers of complicated aggressive disease.

Correlation of serum biomarkers with genotypes and clinical phenotypes would enhance our understanding of the pathophysiology of IBD and lead to new tools for diagnosis and stratification of patients for clinical trials. Biomarkers are helpful in prioritizing further examinations, including endoscopy, and/or in the decision to start or intensify treatment in IBD. CRP has many advantages, but its short half-life makes this a particularly good biomarker in the detection and follow up of disease activity in Crohn's disease. In contrast, ulcerative colitis (with the exception of severe colitis) has only a modest-to-absent CRP response despite active inflammation. As stools are easy accessible in IBD patients, fecal biomarkers hold a specific promise and recent studies even claim superiority of fecal biomarkers over serum markers. A number of neutrophil-derived proteins shedding in stools have been studied. Calprotectin and lactoferrin are probably the most promising given their abundance in granulocytes and their stability and resistance to degradation. Although calprotectin and lactoferrin are very sensitive markers to detect inflammation in the gastrointestinal tract, they are not specific for IBD and increased levels are also found in neoplasia, NSAID abuse, infections and polyps. In children with abdominal symptoms and diarrhea, a positive test for calprotectin or lactoferrin may prioritize endoscopy.

In 2012, Genisphere and the Lankenau Institute for Medical Research expanded their research collaboration covering miRNA biomarkers for ulcerative colitis to include Crohn's disease and other IBDs. The partners will use the Affymetrix GeneChip miRNA Array to identify the miRNA biomarkers. The goal is to identify biomarker panels that can classify the various forms of IBD and develop a diagnostic test to shed light on how patients are being correctly or incorrectly diagnosed in their respective diseases and to provide information on how they are responding to treatment. This will enable personalized treatment of IBD.

Biomarkers of Radiation Injury

There is need for discovery of biomarkers that would be used to predict acute and delayed radiation injuries to organs and tissues after a radiological terrorist attack in order plan triage and make medical decisions. Such biomarkers would be

particularly valuable because radiation injuries can take days or weeks before clinical manifestations, and individuals differ in their sensitivity to radiation. For these biomarkers to be useful, they would need to be linked to relevant clinical outcomes, such as organ failure, and it would need to be shown that the change in the biomarker is related to the radiation exposure and not other factors. Tests developed based on these markers should be rapid, reliable, inexpensive, and easy to use. The biomarkers may also provide leads for development of countermeasures against radiological or nuclear attacks. In 2011, the National Institute of Allergy and Infectious Diseases announced plans to fund research that will discover and develop predictive biomarkers for radiation injuries in civilian populations. Investigators may use these grants to develop radiation injury biomarkers for measuring or characterization of gene and protein expression, DNA or protein modifications, metabolomic or lipidomic changes, or cytogenetic, inflammatory, biochemical, and other changes. These biomarkers may also be useful in predicting delayed effects of therapeutic radiation for cancer (see Chap. 6).

A project at the Arizona State University (ASU) in 2013 demonstrated that gene expression is a viable approach to directly measure radiation exposure, including identifying and validating biomarker signatures by providing an accurate indication of the level of absorbed radiation. The project is entering a $9.33 million contract option as part of a 5-year, $35.44 million project funded by the Biomedical Advanced Research and Development Authority within the Office of the Assistant Secretary for Preparedness and Response of the US Department of Health and Human Services. ASU is partnering with Life Technologies (now part of ThermoFisher Scientific) to develop real-time PCR assays based on these gene signatures that would run on several instruments including the Applied Biosystems 7500 Fast Dx and the QuantStudio Dx. Ultimately, the group would need to seek regulatory approval for the assay from the FDA.

Biomarkers for Prediction of All-Cause Mortality

NMR spectroscopy of plasma samples from a random subset of the Estonian Biobank revealed that 4 biomarkers in the blood – alpha-1-acid glycoprotein, albumin, VLDL particle size, and citrate – can be used to assess whether otherwise healthy people are at short-term risk of dying from heart disease, cancer, and other illnesses (Fischer et al. 2014). The biomarker profiling improved prediction of the short-term risk of death from all causes above established risk factors. Further investigations are needed to clarify the biological mechanisms and the utility of these biomarkers for guiding screening and prevention.

Biomarkers Common to Multiple Diseases

Some biomarkers are found in more than one disease and their evaluation requires correlation with clinical manifestations. Some examples are listed in Table 5.3.

Table 5.3 Examples of biomarkers common to multiple diseases

Biomarker	Diseases
Chromogranin A	Neuroendocrine tumors, cardiovascular disease, sepsis,
C reactive protein (CRP)	Diabetes mellitus, sepsis, pulmonary diseases, acute myocardial infarction, renal dysfunction
Cystatin C	Inflammation, myocardial infarction, renal failure, cancer, Alzheimer disease, amyotrophic lateral sclerosis, multiple sclerosis
Natriuretic peptide	Ischemic heart disease, infections
Nitric oxide	Asthma (in breath), acute respiratory distress syndrome (in urine), cardiovascular disease (in plasma)
Oxidative stress biomarkers	Most diseases with oxidative stress
Serum 100B protein	Traumatic brain injury, stroke, epilepsy (in CSF)
Tau protein	Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jakob disease, AIDS encephalopathy, alcohol-induced organic brain disorders
TNF-α	Rheumatoid arthritis (serum and synovial fluid), neuroinflammation, ischemic heart disease

Myositis specific autoantibodies (MSAs) have been already described earlier in this chapter as biomarkers of idiopathic inflammatory myopathies. However, they overlap and clinically correlate with several other disorders.

Nasal Nitric Oxide as a Biomarker of Response to Rhinosinusitis Therapy

The assessment of the response of chronic rhinosinusitis to therapy is difficult. CT scans cannot be used repeatedly. Therefore, methods such as symptom scores and endoscopy are employed instead. The paranasal sinuses and nasal mucosa are a major source of exhaled nitric oxide (NO). Nasal NO (nNO), which is easily measured, provides a valuable non-invasive biomarker of response of chronic rhinosinusitis to therapy. Topical nasal corticosteroids may be needed to reduce the contribution of nasal eNOS and NO emanating from the sinuses can be measured.

A study has compared nNO levels in patients with chronic rhinosinusitis as measured by electroluminescence with those of common cold patients as well as controls and correlated nNO levels with endoscopic and CT findings (Dabholkar et al. 2014). The measured levels of NO did not differ between healthy volunteers and common cold patients, but they were significantly lower in patients suffering from chronic rhinosinusitis. As NO is a regulator of mucociliary activity and has bacteriostatic and antiviral effects, the decreased concentration of nNO in patients suffering from sinusitis suggests that lack of NO may contribute to the pathogenesis of this disease. Thus, nNO is a valuable objective measure of chronic rhinosinusitis. However, the expense of NO chemiluminescence analyzers may limit application of the method in clinical practice.

Biomarkers of Gene-Environmental Interactions in Human Disease

Gene-environmental interactions play an important role in human disease, but they have not been studied systematically. The Genes, Environment and Health Initiative (GEI) of NIH was announced in 2006 to support research that will lead to the understanding of genetic contributions and gene-environment interactions in common disease. GEI is being developed and planned by an NIH-wide Coordinating Committee, administratively led by the National Human Genome Research Institute (NHGRI) and the National Institute of Environmental Health Sciences (NIEHS). NIH GEI has awarded a $5.9 million grant to the Pacific Northwest National Laboratory (PNNL) that will house the Center for Novel Biomarkers of Response. Scientists at the center intend to create new exposure assessment tools to better understand the role of gene-environmental interactions in human disease. Development of these tools will enable precise measurements of personal exposure to environmental, chemical and biological agents. Two of the most important risk factors for human morbidity and mortality – cigarette smoke and obesity – will be the primary targets of interest for PNNL scientists. Research will focus on biomarkers for systemic stress caused by mainstream and second-hand cigarette smoke, with obesity as a confounding physiological factor. This research is comprised of two basic elements. The genetic component will rely on newfound abilities to swiftly identify genetic differences between people with illnesses and those who are healthy, leading to a greater understanding of genetic contribution to the disease. The environmental biology component will focus on developing new technologies to accurately measure personal exposures with small, wearable nanobiosensors that can be used to assess environmental agents. The center will provide NIH NIEHS with a database of response biomarkers, as well as chemical substances for selected biomarkers that are tested and validated in humans and supported by parallel studies in mice. The researchers will also develop nanobio sensors for measurement of biomarkers at POC.

Application of Biomarkers in Animal Health

Many of the advances in human healthcare are being applied to animal health. Some of the new technologies were tested in experimental animals before they were introduced into human medicine. Biomarkers are studied in animal models of human diseases. Table 5.4 shows some examples of biomarkers used in animal healthcare.

Dogs suffer from many of the same disease as humans. The value of measuring blood levels of the myocardial protein cardiac troponin I (cTnl) has been tested for the diagnosis of congenital and acquired heart disease in the dog and in the evaluation of the severity of heart failure. Serum samples obtained from healthy dogs and from dogs diagnosed with a variety of congenital and acquired heart conditions can

Table 5.4 Examples of use of biomarkers in animal health

Biomarker	Relevant clinical conditions
Biomarkers of cartilage degradation and synthesis measured by ELISA assays	Canine osteoarthritis
Biomarkers of endothelial cell activation	Drug-induced vascular injury
Cardiac troponin I	Heart failure in dogs
Cross-sectional brain imaging	CNS trauma and infections
Telomeres lengths and expressed telomerase activity in lens epithelium	Canine cataract

be assayed for cTnl concentration using an automated immunoassay method. Results can also analyzed according to the degree of heart failure as assessed using the International Small Animal Cardiac Health Council's scheme. Healthy dogs have very low or undetectable blood cTnl levels, as do dogs with congenital heart disease. However, cTnl levels are significantly elevated in dogs with acquired mitral valve disease, dilated cardiomyopathy and pericardial effusion. Blood cTnl levels also vary with severity of heart failure. Measurement of blood cTnl levels may be a useful aid in the diagnosis of dogs with suspected heart disease and in indicating the severity of heart failure.

Cataracts in small animals are shown to be at least partially caused by oxidative damage to lens epithelial cells (LECs) and the internal lens. Therefore, biomarkers of oxidative stress in the lens are considered as general biomarkers for life expectancy in the canine and other animals. Telomeres lengths and expressed telomerase activity in canine LECs may serve as important monitors of oxidative damage in normal LECs with documented higher levels of telomerase activity in cataract. Loss of functional telomere length below a critical threshold in LECs of canines during the effect of UV and chronic oxidative stress or metabolic failure, can activate programs leading to LEC senescence or death. Telomerase is induced in LECs of canines at critical stages of initiation of cataract pathogenesis and exposure to oxidative stress through the involvement of transition metal ions such as, ferrous ions-catalytic oxidants (Babizhayev and Yegorov 2014). Early detection is important as treatment with 1% N-acetylcarnosine lubricant eye drops is beneficial for prevention and dissolution of ripe cataracts in canines.

Cross-sectional imaging techniques have facilitated diagnosis of central nervous system (CNS) diseases in companion animals. However, there is still frequently a lack of definition of the cause of neurologic lesions, because tissue sampling from the pathologic site is often difficult and there are few clinical diagnostic tools to assist diagnosis. Biomarkers can assist in understanding the cause, diagnosis, severity, and prognosis for neural injury. Integration of conventional testing and new diagnostic techniques will overcome shortcomings in understanding infectious diseases of the CNS. Diagnostic tests may be limited by poor positive and negative predictive values, which must be recognized when interpreting test results (Nishida 2014).

FDA researchers are trying to find and identify biomarkers for inflammatory diseases in cows, as well as proteins that could serve as alternatives to antibiotics for use in food animals. The goal is to develop tools that will allow the FDA to regulate new drugs for diseases for which no treatments exist, or better treatments than currently exist.

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Chapter 6
Biomarkers in Metabolic Disorders

There is considerable information available about biomarkers of metabolic disorders. The use of some of these biomarkers is well established. Coverage of all metabolic disorders is beyond the scope of this report. Example of acute intermittent porphyria, liver X receptors, diabetes and the metabolic syndrome will be discussed in this section.

Biomarkers of Acute Intermittent Porphyria

Acute intermittent porphyria (AIP) is a metabolic disease caused by a deficiency of hydroxymethylbilane synthase, which affects hepatic heme biosynthesis. Clinical manifestations are abdominal pain and neurovisceral symptoms, accompanied by overproduction of heme-precursors in the liver, which frequently remains long-lasting in AIP patients. Treatment is based on symptomatic relief together with carbohydrate loading and in more severe attacks heme therapy. During an acute attack the heme precursors porphobilinogen (PBG) and 5-aminolevulinic acid (ALA) are produced in high amounts by the liver and are found in high concentrations in plasma and urine. These metabolites represent the acute phase reactants confirming an ongoing attack and are used to evaluate therapeutic measures. Biochemical monitoring of an acute attack is more accurately reflected by plasma PBG than plasma ALA or urinary PBG and ALA (Sardh et al. 2009).

AIP may be associated with alterations of hepatic proteins known to be either increased or decreased in serum according to diverse pathological conditions including malnutrition, inflammation or liver disease. Most of the serum proteins are within normal limits in these patients, however insulin-like growth factor 1 (IGF-1) is decreased in 53.8% of AIP patients and transthyretin (prealbumin) is found significantly decreased in 38.5% of them (Delaby et al. 2009). The use of both IGF-1 and transthyretin has been proposed as biomarkers of disease morbidity/severity for the clinical follow-up of AIP patients.

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Liver X Receptors

The liver X receptors (LXRs) are nuclear receptors that play central roles in the transcriptional control of lipid metabolism and are useful biomarkers of metabolic disorders. LXRs function as nuclear cholesterol sensors that are activated in response to elevated intracellular cholesterol levels in multiple cell types. Once activated, LXRs induce the expression of an array of genes involved in cholesterol absorption, efflux, transport, and excretion. In addition to their function in lipid metabolism, LXRs have also been found to modulate immune and inflammatory responses in macrophages. Synthetic LXR agonists promote cholesterol efflux and inhibit inflammation in vivo and inhibit the development of atherosclerosis in animal models. The ability of LXRs to integrate metabolic and inflammatory signaling makes them particularly attractive targets for intervention in human metabolic disease. LXR agonists prevent the development and progression of atherosclerosis and inhibit neointima formation following angioplasty of the arterial wall. Data from studies on mouse liver suggest that nuclear receptor transcriptome is modulated in proliferating liver and is a source of biomarkers and pharmacological targets for the management of liver disease affecting hepatocyte proliferation (Vacca et al. 2014).

Biomarkers of Diabetes Mellitus

Diabetes mellitus results from abnormal function of pancreatic β cells – specialized structures that produce insulin. Type 1 diabetes (insulin dependent) generally results from autoimmune destruction of pancreatic islet β cells. Type 2 diabetes mellitus (non-insulin dependent) is caused by the failure of the pancreatic β cells to secrete sufficient insulin to compensate a decreased response of peripheral tissues to insulin action. LC/MS followed by multivariate statistical analysis has been successfully applied to the plasma phospholipids metabolic profiling in type 2 diabetes mellitus. It is a complement or an alternative to NMR for metabonomics applications. Diabetes has a causative role in many diseases and its complications affect several organs. Biomarkers of diabetes mellitus are shown in Table 6.1.

β-Cell Function as Biomarker of Diabetes

β-cells are known vanish prior to the development of type 2 diabetes mellitus (T2DM), and autopsy of overt T2DM patients have shown a 60% reduction in β-cell mass. As the decline in β-cell function and mass have been proven to be pathological traits in T2DM, methods for evaluating β-cell loss is becoming of more interest. However, evaluation of β-cell death or loss is currently invasive and unattainable for the vast majority of diabetic patients. Serological biomarkers, reflecting β-cell loss

Table 6.1 Biomarkers of diabetes mellitus

Biomarkers of hyperglycemia
Increased serum free fatty acids
Increased ketones
Exhaled methyl nitrate
Biomarkers of diabetes-associated oxidative stress
Elevated serum malondialdehyde, lipid hydroperoxides, and lipoperoxides
Elevated levels of plasma thioredoxin
Elevated superoxide dismutase in RBCs
Elevated plasma protein carbonyl levels
Increased urinary 8-hydroxy-2′-deoxyguanosine
Biomarkers of inflammation
C-reactive protein (CRP)
Plasma-soluble cell adhesion molecules
Monocyte IL-6
Nitrotyrosine
Biomarkers of renal complications in type 2 diabetes mellitus
Apolipoprotein B: elevated
Creatinine: elevated
Glycosylated albumin
Low-density lipoprotein: elevated
Soluble tumor necrosis factor receptor: elevated
Triglycerides: elevated
Biomarkers of endothelial dysfunction in type 2 diabetes mellitus
E-selectin
Intercellular adhesion molecule 1
Vascular cell adhesion molecule 1
Biomarkers of insulin resistance
Increase of sugar metabolites: increase of N-acetylglucosamine, deoxyglucose and glucuronic acid
Serum retinol binding protein 4
Biomarkers of diabetes with cardiovascular complications
Adiponectin
Disturbances of lipid metabolism: decreased medium chain fatty acids, increased longer chain fatty acids
Glycosylated hemoglobin
Genes associated with diabetes
G6PC2 gene polymorphisms associated with elevated fasting plasma glucose
IRS1 as a diabetes susceptibility gene is linked to insulin resistance
KIAA0350 (a sugar-binding, C-type lectin) as risk factor for diabetes type 1
Rare MTNR1B variants which impaired melatonin receptor 1B function contribute to type 2 diabetes
Serum biomarkers of β-cell function

could detect and monitor progression of T2DM non-invasively. Biomarkers with such capacities could be neo-epitopes of proteins with high β-cell specificity containing post-translational modifications. Such tools may segregate T2DM patients into more appropriate treatment groups, based on their β-cell status, which is currently not possible. Presently individuals presenting with adequately elevated levels of both insulin and glucose are classified as T2DM patients, while an important subdivision of those is pending, namely those patients with sufficient β-cell capacity and those without. This may warrant two very different treatment options and patient care paths. Serological biomarkers reflecting β-cell health status may also assist development of new drugs for T2DM and aid physicians in better characterization of individual patients and tailor individual treatments and patient care protocols (Neutzsky-Wulff et al. 2012).

Biomarkers of Hyperglycemia

Metabolic characteristics of hyperglycemia in the diabetic such as low insulin and increased free fatty acids and ketones in serum are well known. Exhaled methyl nitrate correlates specifically with acute, spontaneous hyperglycemia of type 1 diabetes mellitus and can be used as a noninvasive biomarker. Gas analysis can be performed on breath samples via gas chromatography using electron capture, flame ionization, and mass selective detection. The kinetic profile of exhaled methyl nitrate, commonly present in room air in the range of 5–10 parts per trillion, strongly correlates statistically with that of plasma glucose. In healthy subjects, exhaled methyl nitrate concentrations are slightly greater than room air concentration, indicating a small net output of this gas by the human body. The biochemical production of this gas has not been postulated; however, the results of this study show that oxidative processes play a major role in its production.

Biomarkers of Diabetes-Associated Oxidative Stress

Erythrocyte glutathione peroxidase activity, glutathione content, and plasma beta-carotene were significantly lower in diabetic patients compared with control subjects, but with no significant differences between patients in whom the disease is controlled and those in whom it is not. Antioxidant enzyme superoxide dismutase (SOD) activity is significantly higher in the erythrocytes of diabetic patients independently of the presence of microvascular complications. However, the plasma alpha-tocopherol/total lipids ratio is significantly diminished in controlled diabetes group compared with uncontrolled diabetes group. Lipid peroxidation indices measured in plasma include malondialdehyde, lipid hydroperoxides, and lipoperoxides, which are significantly elevated in diabetic patients regardless of the presence of complications. Evidence of oxidative damage to proteins can be shown by elevation of plasma protein carbonyl compared. Additionally, a marked increase in protein oxidation is observed.

Biomarkers of Inflammation Associated with Diabetes

Type 1 diabetes is associated with increased vascular complications, and monocytes are pivotal cells in atherogenesis. Biomarkers of inflammation – C-reactive protein, plasma-soluble cell adhesion molecules, monocyte IL-6, and nitrotyrosine – are significantly elevated in type 1 diabetic subjects compared with in control subjects. These findings have a major implication on our understanding of the role of inflammation in vasculopathies in type 1 diabetes.

Biomarkers of Renal Complications in Diabetes Mellitus Type 2

Dyslipidemia and inflammation may promote renal disease in type II diabetes mellitus. Several potentially modifiable lipid and inflammatory biomarkers – triglycerides, low-density lipoprotein, apoprotein B, fibrinogen, soluble TNFR and vascular cell adhesion molecule-1 – are elevated in the setting of moderately decreased GFR in men with type 2 diabetes mellitus and may be the link between renal insufficiency and increased risk for cardiovascular events in this population. Clinical and laboratory use of endothelial, inflammatory and pro-coagulant biomarkers for predicting the risk of cardiovascular and renal complications in diabetic patients and for monitoring these patients is promising (Domingueti et al. 2016).

Biomarkers of Diabesity

Diabesity is the term used for the syndrome of gradual progression of obesity to type 2 diabetes with overlapping symptoms of insulin resistance, hyperinsulinemia, hyperglycemia, dyslipidemias, ion imbalance, and inflammation. The Busselton study (http://bpmri.org.au/), in progress in Australia, is trying to identify biomarkers in the blood that will predict the onset of diabetes before clinical symptoms manifest. The study is being conducted by Busselton Population Medical Research Institute in Australia.

Biomarkers of Prediabetes

According to 2011 figures from the Centers for Disease Control and Prevention (CDC), 8.3% of the US population, i.e. 25.8 million, have diabetes, of which ~7 million are unaware they have the disease. Based on glycemic measures from 2005–2008, the CDC reported that 35% of the US adult population had pre-diabetes, yielding an estimated 79 million US adults at risk for developing type 2 diabetes. Tests for detection of prediabetes are being developed that are based on biomarkers.

Metabolon Inc has discovered a number of significant biomarkers indicative of risk for type 2 diabetes, which are being incorporated in the development of the Quantose™ series of diagnostic products. The first-generation Quantose™ product is a diagnostic test for better identifying high-risk, insulin-resistant patients and predicting their risk of progression to type 2 diabetes, above and beyond traditional risk factors and glycemic tests. The validated test is currently being offered in Metabolon's CLIA laboratory as "Investigational Use Only".

PreDx™ Diabetes Risk Test (Tethys Bioscience), based on a set of protein biomarkers, is a predictive tool for an accurate assessment of an individual's risk of developing type 2 diabetes within the next 5 years. The test is performed exclusively by the Tethys' Clinical Laboratory on routinely collected blood samples.

Biomarkers of Insulin Resistance

Insulin receptor deficiency is the key link in the insulin resistance and has a causal role in type 2 diabetes. Adipokinins including adiponectin, leptin, resistin, IL-6, PPARγ and TNF-α play an important role in insulin resistance.

Serum levels of retinol-binding protein 4 (RBP4), a protein secreted by adipocytes, are increased in insulin-resistant states. Transgenic overexpression of human RBP4 or injection of recombinant RBP4 in normal mice causes insulin resistance. RBP4 is elevated in the serum before the development of frank diabetes and is a biomarker for identifying insulin resistance and associated cardiovascular risk factors in subjects with varied clinical presentations. Exercise training is associated with a reduction in serum RBP4 levels only in subjects in whom insulin resistance improves. RBP4 levels can be normalized by rosiglitazone, an insulin-sensitizing drug. Adipocyte GLUT4 protein and serum RBP4 levels are inversely correlated. These findings provide a rationale for antidiabetic therapies aimed at lowering serum RBP4 levels.

A high-throughput 92-protein assay was used to identify circulating biomarkers of insulin resistance in two cohorts of community residents without diabetes (Nowak et al. 2016). Proteomic blood profiling indicated cathepsin D as a new biomarker of insulin resistance and suggested a causal effect of insulin resistance on tissue plasminogen activator.

Glycosylated Hemoglobin in Diabetes Mellitus

Combining fasting plasma glucose and glycosylated hemoglobin (HbA1c) improves the accuracy for detecting patients with diabetes mellitus type 1. Combination of HbA1c level and OGTT enables more precise prediction of progression to diabetes

mellitus type 2 in subjects with glucose intolerance. The glycosylated hemoglobin value is the primary target for glycemic control. The American Diabetes Association recommends that the blood test — which measures the average level of glycemia over the preceding 2–3 months — be performed at least twice a year in patients whose treatment goals are being met (and who have stable glycemic control) and quarterly in patients whose treatment has changed or whose goals are not being met. In persons with diabetes, chronic hyperglycemia, as assessed by HbA1c level, is associated with an increased risk for cardiovascular disease. Limitations of HbA1c as a biomarker of diabetes are:

- It is inaccurate for patients on hemodialysis and for patients with red blood cell disorders
- Performed only 2–4 times per year; not frequent enough to detect the advance of diabetes complications in a timely manner
- Poor physician-patient feedback; the test is usually processed by an outside laboratory so results are reported to the doctor at a later date.

Glycated Albumin as a Biomarker of Diabetes Mellitus

Albumin, a serum protein in the blood, can be measured precisely and has a turnover time of 2–3 w. Like many proteins in the body, albumin can become altered or glycated. High levels of glycated albumin have been directly linked to major complications of diabetes such as retinopathy and nephropathy by damage caused to small blood vessels by the altered protein. It is also a biomarker for other types of diabetic complications. More than 25 years of clinical research has proven that monthly measurement of glycated albumin is a superior technology to monitor and control glycation.

Epinex G1A™ Rapid Diabetes Monitoring Index Test (Epinex Diagnostics Inc) is a monthly test for the control of glycation. The test is composed of the G1A™ reader and a proprietary dual-channel test cassette, which is able to simultaneously test for glycated albumin and total albumin. The G1A™ reader automatically quantifies the analyte concentrations and gives the G1A™ Index, the ratio of glycated albumin to total albumin in serum, which shows how well the patients have controlled their level of glycation over the previous month. Simple to operate, the G1A™ test system can be performed at the point-of-care without the need for highly trained laboratory technicians. It enables immediate feedback between healthcare provider and patients, thus facilitating timely therapeutic interventions. The results are stored on the device, providing trend analysis as well as displaying immediate results. The results can be transmitted by means of a computer or wireless connection to a doctor's office or other central data location.

Low C-Peptide as a Biomarker of Complications of Diabetes Type 1

Pancreatic β cells produce proinsulin, which splits into C-peptide and insulin, and both are released into circulation. C-peptide binds to a receptor at the cell surface and activates signal transduction pathways that result in stimulation of Na^+, K^+-ATPase, and endothelial nitric oxide synthase (eNOS), both of which are enzymes with reduced activities in diabetes. In type 1 diabetes patients no proinsulin is produced and neither insulin nor C-peptide is formed. A cross-sectional study has shown that low C-peptide levels have clinical significance and appear helpful in characterizing groups at-risk for faster C-peptide decline, complications, poorer metabolic control and severe hypoglycaemia (Kuhtreiber et al. 2015). Low C-peptide levels may be a biomarker for characterizing at-risk patients with Type 1 diabetes. C-peptide studies by using surface plasmon resonance (SPR) and electrospray mass spectrometry (ESI-MS) show how proinsulin is found to influence insulin-insulin interactions. These technologies can be used for measurements of C-protein as a biomarker.

Lack of C-protein is a biomarker of early peripheral neuropathy in diabetes before painful neuropathy develops with structural damage to the nerve. Replacement of C-peptide in clinical trials by Creative Peptides has been shown to improve the early signs of peripheral neuropathy such as sensory impairment. There is concomitant improvement of renal dysfunction (normalized glomerular filtration, decreased albumin excretion) and reduces diabetes-induced structural changes (decreases mesangial expansion) after C-peptide replacement.

Personalized Management of Diabetes Mellitus Based on Biomarkers

Gene, protein, and metabolite biomarkers provide new insights into pathophysiology of type 2 diabetes mellitus and each of them can identify useful biomarkers for personalized medicine of this disease (Chen and Jia 2012). However, the combination of these three types of biomarkers has not been applied for personalized management of diabetes as yet. Also, the role of interaction between genes and environment in the pathogenesis of diabetes is not clear. It is possible in the future that molecular biomarkers will be used simultaneously to direct treatment decisions, but further investigations are needed. For possible biomarkers to be validated, meaningful improvement needs to be demonstrated in the prediction of various events or complications in the course of the disease.

Biomarkers of Metabolic Syndrome

The metabolic syndrome (MetS), which is very common in the general population, is defined by the clustering of several classic cardiovascular risk factors, such as type 2 diabetes, hypertension, high triglycerides and low high-density lipoprotein cholesterol (HDL). Central obesity and insulin resistance, which are the two underlying disorders of the syndrome, are further risk factors for cardiovascular disease. Novel risk factors of the MetS include biomarkers of chronic mild inflammation, increased oxidant stress, high cystatin C levels, thrombophilia and endothelial dysfunction. Therefore, subjects with the MetS are potentially at high risk of developing atherosclerosis and clinical cardiovascular events. Several longitudinal studies have confirmed that subjects with the metabolic syndrome present with atherosclerosis and suffer from myocardial infarction and stroke at rates higher than subjects without the syndrome. The risk of cardiovascular disease is particularly high in women with the syndrome and in subjects with pre-existing diabetes, cardiovascular disease and/or high CRP. However, an increased risk is already present in subjects with a cluster of multiple mild abnormalities. The risk related to the metabolic syndrome is definitely higher when subjects affected are compared to subjects free of any metabolic abnormality.

Adiponectin

Adiponectin, secreted by adipocytes, accounts for ~0.01% of total plasma protein. Unlike other adipocyte products, adiponectin correlates with decreased free fatty acid blood concentrations and reduced body mass index or body weight. It has been well established to be an important biomarker for MetS and its complications. Animal and cell culture experiments also support most claims from human observations of its roles in the MetS. Reproducible results of human genetic studies of diverse ethnic origin and by different investigators may provide the evidence for its causative roles in the pathogenesis of the MetS and further insight into the genetic constitutions of the MetS. Some of the common polymorphisms in the promoter region, exon and intron 2, and the rare nonsynonymous mutations in exon 3 of the human adiponectin gene were repeatedly shown in many studies from many different ethnic populations to associate with the phenotypes related to body weight, glucose metabolism, insulin sensitivity, and risk of type 2 diabetes mellitus and coronary artery disease. The common polymorphisms and rare mutations of the human adiponectin gene are associated with differential expression of adiponectin at the plasma protein level and mRNA level in adipose tissue. The PPARγ2 Pro12Ala variants also influence insulin sensitivity in interaction with adiponectin genotype or influence plasma adiponectin levels. Human genetic studies on adiponectin and the metabolic syndrome strongly suggest that adiponectin is one of the causative

factors in its pathogenesis and provide significant insights into the genetic makeup of the MetS. Extension from these studies may accelerate the discovery of new molecular targets for future therapeutic interventions.

Clinical studies have confirmed that treatment with thiazolidinediones may increase adiponectin concentrations in patients with type 2 diabetes independent of improvements in blood glucose control or parallel treatment with insulinotropic drugs. Thus, adiponectin may have a diagnostic value and can be used for monitoring treatment.

Cystatin C

Cystatin C is a biomarker of cardiovascular disease, and a study has prospectively investigated whether plasma levels of cystatin C predict new-onset MetS as well as long-term progression and incidence of the different components of the MetS (Magnusson et al. 2013). Cystatin C was measured in individuals included in the Malmö Diet and Cancer cardiovascular cohort who were free from the MetS at baseline and subsequently underwent a follow-up examination after a median period of 16 years. MetS was defined according to the NCEP-ATP-III guidelines. During follow-up, 428 out of 1502 subjects developed new-onset MetS. In age- and sex-adjusted analysis, compared to the lowest quartile of cystatin C, the odds ratios for incident MetS in subjects with cystatin C levels in quartiles 2, 3 and 4 were 1.00, 1.48 and 1.91, respectively,; this linear association remained significant even after full multivariate adjustment. In this fully adjusted model, long-term progression of abdominal obesity was the only component of the MetS significantly associated with increasing quartiles of baseline cystatin C levels. These findings suggest that cystatin C may adversely affect metabolic factors, particularly abdominal obesity, thus contributing to development of the MetS.

Human Plasma Lipidome

The increase in prevalence of metabolic diseases, including type 2 diabetes and obesity, which are associated with an elevated risk of cardiovascular disease, requires more detailed lipid analyses both for diagnostic purposes and for monitoring the efficacy of prescribed therapy. Lipids in plasma are solubilized and dispersed through their association with specific groups of proteins.

Lipids circulate in the blood as lipoprotein particles, including chylomicrons, very-low-density lipoprotein (VLDL), lowdensity lipoprotein (LDL), and high-density lipoprotein (HDL). In circulating chylomicrons and VLDL, triglycerides undergoes hydrolysis, catalyzed by lipoprotein lipase (LPL), to generate a pool of free fatty acids (FFAs) that is used as an energy source in tissues, including muscle. Excess FFAs are stored in adipocytes. Such caloric abundance leads to an unopposed expansion of adipose tissue and, ultimately, to obesity and associated

metabolic complications characterized by insulin resistance and diabetes. Stored triacylglycerol in adipocytes undergoes lipolysis on demand as a result of hormone-sensitive lipase, leading to an energy-balanced level of FFAs in plasma. Saturated FFAs promote cardiac disorders and systemic inflammation, whereas n−3 FFAs prevent these effects. LDL-derived cholesterol, in both its free form (FC) and its esterified form (CE), contributes to the development of cardiovascular disease. HDL helps remove excess FC by reverse cholesterol transport, with the formation of CE by lecithin cholesterol acyltransferase, and subsequent uptake of the CE by the liver. High levels of HDL are correlated with low cardiovascular risk (Quehenberger and Dennis 2011).

Neurotensin as Biomarker of Obesity

Neurotensin (NT), a 13-amino-acid peptide predominantly localized in specialized enteroendocrine cells of the small intestine and released by fat ingestion, facilitates fatty acid translocation in the intestine, and stimulates the growth of various cancers. The effects of NT are mediated through 3 known NT receptors (NTR1, 2 and 3). Increased fasting plasma levels of pro-NT (a stable NT precursor fragment) are associated with increased risk of diabetes, cardiovascular disease and mortality. An experimental study has shown that NT-deficient mice demonstrate significantly reduced intestinal fat absorption and are protected from obesity, hepatic steatosis and insulin resistance associated with high fat consumption (Li et al. 2016). The authors further demonstrate that NT attenuates the activation of AMP-activated protein kinase (AMPK) and stimulates fatty acid absorption in mice and in cultured intestinal cells, and that this occurs through a mechanism involving NTR1 and NTR3 (also known as sortilin). In humans, both obese and insulin-resistant subjects have elevated plasma concentrations of pro-NT, and in longitudinal studies among non-obese subjects, high levels of pro-NT denote a doubling of the risk of developing obesity later in life. These findings directly link NT with increased fat absorption and obesity and suggest that NT may provide a prognostic biomarker of future obesity and a potential target for prevention and treatment. Sphingotec GmbH's Sphingotest® pro-NT uses Pro-NT as a stable surrogate biomarker for neurotensin. Elevated fasting plasma levels of pro-NT are associated with increased risk of obesity, cardiovascular diseases, and breast cancer.

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Chapter 7
Biomarkers in Immune Disorders

Introduction

There are a large number of immune disorders that include rheumatoid arthritis, multiple sclerosis (see Chap. 8), type 1 diabetes (Chap. 6), systemic lupus erythematosus, and psoriasis. Some of these have inflammation as well. Many of these conditions are characterized by the expression of cell surface biomarkers and cytokines produced by T and B lymphocytes, which can be used to as an indicator of the disease and to monitor patient's response to therapy. Rejection of allografts also involves immune mechanisms and there is an interest in finding predictive biomarkers of organ rejection or tolerance after transplantation. As most of the current biomarkers are based on tissues and cells, another area of investigation is to find plasma biomarkers.

Biomarkers Relevant to Organ Transplantation

The human leukocyte antigens (HLA) encoded by genes within the major histocompatibility complex display an impressive degree of polymorphism. HLA markers are proteins found on the surface of certain cells in the body. They are used by the body's immune system to identify material that is foreign, such as viruses or bacteria. HLA antibody identification is important for organ transplant donor-recipient matching because, in the case of organ donation, a patient's immune system may fight cells from the donor, causing organ failure or rejection. DynaChip HLA Antibody Analysis System (ThermoFisher Scientific) is the only approved automated chip-based system for HLA antibody detection and identification.

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Biomarkers of Graft Versus Host Disease

Following transplantation of major organs such as heart, kidney, and liver, rejection of grafted organs is an important problem. There is a need for non-invasive tests to monitor these patients for adjusting their immunosuppressive drug treatment and early detection of rejection. There is a need for discovery of predictive biomarkers for these patients.

Gene-expression signatures have been studied in peripheral blood mononuclear cells isolated from patients with graft versus host disease (GVHD) as well as immunosuppressed transplant recipients. Blood leukocyte expression signatures may be applied for assessment of immune status and early detection of GVHD. For clinical trials in chronic GVHD, the following applications of biomarkers may be useful:

- Predicting response to therapy.
- Measuring disease activity and distinguishing irreversible damage from continued disease activity.
- Predicting the risk of developing chronic GVHD.
- Diagnosing chronic GVHD.
- Predicting the prognosis of chronic GVHD.
- Evaluating the balance between GVHD and graft-versus-leukemia effects (graft-versus-leukemia or GVT)
- Serving as a surrogate end point for therapeutic response.

With the advancement of many high-throughput ‘omic techniques such as genomics, proteomics, and metabolomics, efforts have been made to understand potential mechanisms of specific graft injuries and develop novel biomarkers for acute as well as chronic rejection (Sarwal 2009). Microarrays are being increasingly used to identify specific patterns of gene expression that predict and characterize acute and chronic rejection, and to improve the understanding of the mechanisms underlying organ allograft dysfunction. It is feasible to develop minimally invasive, rapid tests for prognosis and diagnosis in personalized management of transplantation patients.

Rashes are very common in patients after bone marrow transplants. They may signal the onset of acute GVHD. But until now, a skin biopsy was the only reliable way for doctors to determine whether the rash is caused by antibiotics commonly used to treat bone marrow transplant patients, or is instead GVHD of the skin, where the disease appears in about half of cases. A large-scale quantitative proteomic discovery procedure was used by University of Michigan scientists to identify biomarker candidates of skin GVHD and validated the lead candidate, elafin, with ELISA (Paczesny et al. 2010). Elafin was overexpressed in GVHD skin biopsies. Plasma concentrations of elafin were significantly higher at the onset of skin GVHD, correlated with the eventual maximum grade of GVHD, and were associated with a greater risk of death relative to other known risk factors. Therefore, elafin has significant diagnostic and prognostic value as a biomarker of skin GVHD. The test, which will be available to clinicians soon, will make informed treatment possible.

This test can determine the risk a patient may have for further complications, and thus physicians will be able to adjust therapy to the degree of risk, rather than treating every patient in exactly the same way.

Biomarkers of Renal Allograft Failure

Survival of renal allografts is limited by chronic allograft deterioration resulting from processes that are difficult to detect in their early stages, when therapeutic interventions would be most effective. Long-term graft loss is still a major problem in renal transplantation. The occurrence of acute rejection episodes and impaired response of the patient to anti-rejection therapy can lead to adverse graft outcome in both the short term and the long term. Currently, clinical featuress and morphologic assessment of the renal biopsy serve as the basis for assessment of risk for graft failure. Molecular biomarkers have been tested for the following purposes: monitoring for acute rejection; identifying steroid-resistant rejections; and monitoring for clinical transplant tolerance. Predictive biomarkers from easily accessible specimens, such as blood or urine, might improve early diagnosis of graft-damaging processes and help with the identification of patients at particularly high risk of sustained injury, thereby helping to tailor therapy and appropriate follow-up screening.

Despite a large number of studies for the prediction of renal allograft failure, none of the investigated candidate biomarkers is robustly established for widespread clinical use or able to replace biopsies for graft assessment (Boenisch and Chandraker 2008). The combination of molecular analyses and cellular immunological assays is essential for undisputed detection of rejection, reversibility of the rejection by therapy, absence of rejection, and long-term graft outcome (Eikmans et al. 2008).

PlexMark™ 3 Renal Biomarker Panel Assay (ThermoFisher Scientific) is a non-invasive and cost-effective research tool for performing kidney function post-transplantation studies rapidly and easily. It uses Luminex® xMAP®, multiplexing technology for bioassay analysis, in a standard immunoassay format to offer ease-of-use, sensitivity and rapid, reproducible results. It measures levels of cytokines, chemokines and receptor levels in urine, which enable researchers to better understand immune function and response. This assay provides researchers with an alternative to invasive and expensive procedures, such as biopsies for obtaining kidney tissue samples to enable research into post-transplantation kidney function.

Quantification of miRNAs in primary cultures of human renal epithelial cells (HRECs) has shown that miR-30a-3p, −10b, and let-7c are highly expressed in HRECs, and that stimulation results in a decreased expression of miR-30a-3p (Anglicheau et al. 2009). These studies, in addition to suggesting a cellular basis for the altered intragraft expression of miRNAs, propose that miRNA expression patterns may serve as biomarkers of human renal allograft status.

A molecular risk score to predict renal graft loss has been derived from analysis of gene expression by microarray examination of biopsies taken between 1 and 31 years after renal transplantation (Einecke et al. 2010). Genes associated with

graft failure were related to tissue injury, epithelial dedifferentiation, matrix remodeling, and TGF-β effects and showed little overlap with rejection-associated genes. Molecular risk score was correlated with interstitial fibrosis, tubular atrophy, tubulitis, interstitial inflammation, proteinuria, and glomerular filtration rate. In multivariate analysis, molecular risk score, peritubular capillary basement membrane multilayering, arteriolar hyalinosis, and proteinuria were independent predictors of graft loss. In an independent validation set, the molecular risk score was the only predictor of graft loss. Thus, the molecular risk score reflects active injury and is superior to either scarring or function in predicting graft failure.

Use of Integrated RNA Data Driven Proteomics (IRDDP) method to assess microarray data from studies involving kidney and heart transplant recipients revealed that three of the proteins – the pro-inflammatory proteins CXCL9, CD44, and PECAM1 – were present at higher levels in the blood of kidney transplant recipients with acute organ rejection (Chen et al. 2010). PECAM1 biomarker could detect acute rejection with 89% sensitivity and 75% specificity, CXCL9 biomarker had 78% sensitivity and 80% specificity, whereas the sensitivity and specificity for CD44 were 80% and 75%, respectively. These biomarkers belong to a specific inflammatory pathway and follow-up work revealed that the same proteins were also found at higher levels in the heart transplant rejection group. The researchers plan to develop an ELISA-based blood test for acute organ rejection using one or more of the biomarkers with the goal to have a commercially available test within the next 3–5 years.

Biomarkers of Renal Transplant Tolerance

Identifying transplant recipients in whom immunological tolerance is established or is developing would allow an individually tailored approach to their posttransplantation management. A multicenter study was conducted to define specific immunological characteristics that identify the tolerant state (Sagoo et al. 2010). It recruited renal transplant patients from distinct clinical groups from across Europe, focusing on operationally tolerant recipients, defined as stable renal transplant recipients that had ceased all immunosuppressive drugs for more than a year with no increase in serum creatinine. The tolerant cohort collected by the Indices of Tolerance consortium in Europe was used as a training set of renal transplant patients on which a series of bioassays and biomarkers were screened for their ability to detect immunological parameters uniquely associated with the tolerant state. In this set a tolerance signature was identified comprising a set of 10 genes with significantly altered expression, elevated numbers of peripheral blood B and NK cells, diminished numbers of recently activated CD4+ T cells, donor-specific hyporesponsiveness of CD4+ T cells, and a high ratio of FoxP3/α-1,2-mannosidase gene expression in peripheral blood, in relation to the other renal transplant comparator groups. These findings were then validated on an independent test set of renal transplant recipients of similar clinical groups recruited by the Immune Tolerance Network in the US.

In another study on the largest cohort of tolerant kidney transplant recipients, renal allograft tolerance was strongly associated with a B cell signature using several assays (Newell et al. 2010). Both studies have identified a B cell signature associated with operational tolerance and show that it is possible to distinguish drug-free tolerant patients from other groups of renal transplant patients and healthy controls with a high degree of specificity and sensitivity. More importantly, cross-platform biomarker analysis highlights subjects within stable renal transplant groups who display an immunological profile similar to that of tolerant patients and who may therefore benefit from managed drug withdrawal.

Biomarkers of Lung Transplant Rejection

Lung transplant patients face a higher death rate than other organ recipients, with 45% dying within 5 years. Until recently, it was not possible to predict transplant failure, and once the signs of chronic rejection appear it is usually too late to reverse it. The aim is to identify patients at risk of chronic rejection before they have the clinical manifestations. Protein biomarkers are an early sign of lung transplant rejection and give an insight into the physiologic mechanisms of rejection. They may provide a guide to the management of lung transplant patients. The dose of anti-rejection drugs should be increased when the biomarkers appear, and reduced in patients without these early signs of rejection.

Microarrays studies to assess gene expression in bronchoalveolar lavage cell samples from lung transplant recipients with and without acute rejection on simultaneous lung biopsies have shown increased expression during acute rejection of genes involved in inflammation, apoptosis, and T cell activation and proliferation. Studies in murine heterotopic tracheal transplant model of chronic rejection have demonstrated specific patterns of gene expression at defined time points after transplantation in allografts, whereas gene expression in isografts reverted back to that of native tracheas within 2 weeks after transplantation. These studies demonstrate the potential power of microarrays to identify biomarkers of acute and chronic lung rejection. The application of new genetic, genomic, and proteomic technologies is in its infancy, and the microarray-based studies are at an early stage of their application to lung transplantation. The massive amount of data generated per tissue or cell sample has spawned an outpouring of invention in the bioinformatics field, which is developing methods to turn data into meaningful and reproducible clinical inferences.

Biomarkers of GVHD Following Transplantation of Hematopoietic Cells

Transplantation of hematopoietic cells from unrelated donors can cure blood disorders but carries a significant risk of acute GVHD, which is higher when the recipient and donor are HLA-DPB1 mismatched. The HLA-DPB1 regulatory region variant

rs9277534 is associated with HLA-DPB1 expression. A study has shown that the risk of GVHD associated with HLA-DPB1 mismatching was influenced by the HLA-DPB1 rs9277534 expression biomarker (Petersdorf et al. 2015). Among recipients of HLA-DPB1 mismatched transplants from donors with the low-expression allele, recipients with the high-expression allele had a high risk of GVHD.

Plasma Biomarkers of Response to Therapy of GVHD

A study compared 12 biomarkers in plasma obtained a median of 16 d after therapy initiation from patients with a complete response by day 28 after therapy initiation and in plasma obtained from patients with progressive GVHD during therapy (Vander Lugt et al. 2013). The lead biomarker, suppression of tumorigenicity 2 (ST2), had the most significant association with resistance to GVHD therapy and subsequent death without relapse. As compared with patients with low ST2 values at therapy initiation, patients with high ST2 values were 2.3 times as likely to have treatment-resistant GVHD and 3.7 times as likely to die within 6 m after therapy. Patients with low ST2 values had lower mortality without relapse than patients with high ST2 values, regardless of the GVHD grade. Plasma ST2 values at day 14 after transplantation were associated with 6 m mortality without relapse, regardless of the intensity of the conditioning regimen. ST2 levels measured at the initiation of therapy for GVHD and during the first month after transplantation improved risk stratification for treatment-resistant GVHD and death without relapse after transplantation.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) affects more than a million persons in the US and Western Europe. It is a chronic B-cell mediated disease manifested by arthralgias, fever, skin rash and end-stage renal disease. Although considered a prototypic autoimmune disease, the hallmark of SLE is its heterogeneity. Accordingly, manifestations can vary widely from person to person, with the potential involvement of virtually any bodily organ. There is no cure for this disease.

Genetic abnormalities underlying this condition are complicated, with diverse genetic polymorphisms described in different ethnic groups, strongly suggesting that the actual pathology underlying the immunologic disarray might not be the same for each patient. Etiology of SLE includes genetic and environmental factors. ITGAM, integrin αM (complement component 3 receptor 3 subunit) encoding a ligand for intracellular adhesion molecule (ICAM) proteins, is an established SLE susceptibility locus. A study has evaluated the independent and joint effects of genetic variations in the genes that encode ITGAM and ICAM by examining several markers in the ICAM1-ICAM4-ICAM5 locus on chromosome 19p13 and the

ITGAM SNP rs1143679 using a large-scale case-control study of unrelated participants from four ancestry populations (Kim et al. 2012). The findings of this study are the first to suggest that an ICAM-integrin-mediated pathway contributes to susceptibility to SLE.

Adiponectin as Biomarker of SLE

Adiponectin is an adipocyte-derived cytokine that has antiinflammatory properties. Plasma adiponectin levels are increased in patients with renal SLE compared to healthy controls and patients with nonrenal SLE. The urine of patients with SLE and kidney involvement, which was shown previously to contain immunoreactive adiponectin, was reexamined for the presence of specific adiponectin isoforms by nondenaturing gel electrophoresis (Song et al. 2009). High molecular weight adiponectin was found in the urine of patients with active lupus nephritis, and may contribute to the renal inflammation of SLE.

Current Management and Need for Biomarkers of SLE

Only three categories of drugs are currently approved for SLE: corticosteroids, antimalarials, and low-dose aspirin. These are used for symptomatic relief or non-specific immunosuppression. Until recently, antibodies to dsDNA or nuclear antigens such as Sm antigen and phospholipids or measurement of complement activation were used together with clinical scores as indicators of drug efficacy in clinical trials. Clinical scores are not satisfactory as there is a considerable lag period between initiation of treatment and clinical effects. There are two potential biologic drugs, rituximab (anti-CD20) and anti-CD22, but drug approval agencies are unable to assess their real efficacy because reliable biomarkers are not available.

The lack of reliable, specific biomarkers not only hampers clinical management of SLE but also impedes development of new therapeutic agents. Based on available data, several laboratory markers have shown promise as biomarkers for susceptibility, diagnosis, and disease activity. Despite the complexities of the many immunologic pathways that are involved in SLE, biomarkers are emerging to characterize patient subgroups, predict prognosis, indicate the exacerbations and remissions of SLE flares, and serve as endpoints in the determination of the dosing and timing of immune-modulating treatments. Recently several clinical studies have tested new therapies directly targeting B lymphocytes. Flow cytometry of circulating peripheral B lymphocytes have been used to define pathogenic subsets of the disease and assess therapeutic efficacy. Biomarkers for SLE include Fc receptor genes (disease susceptibility), complement C4d-bound erythrocytes (diagnosis or disease activity), CD27 plasma cells (disease activity), ‘interferon signature’ (disease activity), and

anti-C1q antibodies (disease activity and organ involvement). These promising candidate biomarkers need to be validated through rigorous, large-scale multicenter studies. There is still an urgent need for better biomarkers with which to monitor disease activity in patients with SLE.

Role of Collaborative Efforts and Databases of SLE Biomarkers

Large databases and multicenter clinical collaborations are needed to identify and validate biomarkdrs of SLE. Recognizing the urgent need for lupus biomarkers, a Lupus Biomarker Working Group has been initiated in the US to facilitate collaborative efforts aimed at identifying and validating biomarkers for SLE. In the EU, BIOLUPUS group is applying genomics, proteomics to construct translational databases for the identification of biomarkers of clinical utility and develop a centralized EU database for SLE. Thirty-five participants from 13 European countries are involved in this enterprise involving clinicians, immunologists and geneticists. Several publications with international collaborators have described the findings of BIOLUPUS. In 2014, Merck Serono and its US affiliate EMD Serono started collaboration with Pfizer Inc. and the Broad Institute (Cambridge, MA) focused on the genomic biomarkers of SLE and lupus nephritis.

C4d-Bearing Reticulocytes

Abnormal levels of C4d, an activation-derived fragment of complement component C4, are deposited on the surface of erythrocytes from patients with SLE; thus C4d-bearing reticulocytes may serve as biomarkers of disease activity in patients with SLE. CD40L has also been reported to be upregulated in patients with SLE and might be a candidate for SLE biomarker. 2′5′-Oligoadenylate synthetase (OAS) was known previously to be related to SLE and was rediscovered to be involved in type I interferon pathway in SLE by several microarray gene expression studies recently, which show that pattern of OAS isoform expressions, particularly of OASL, may provide useful information in differentiating disease flares from certain infections in SLE.

CB-CAPS

A proprietary technology platform for SLE developed at the University of Pittsburgh Arthritis Institute (Pittsburgh, PA) is based upon measurement of cell-bound complement activation products (CB-CAPS). More than 10,000 assays of CB-CAPs bound to erythrocytes, platelets, and white blood cells have been performed. The data suggest that CB-CAPs may serve as universal biomarkers for diagnosis,

disease activity monitoring, identification of disease subsets in SLE, and identification of those at risk for catastrophic events such as heart attack and stroke. Initial studies were conducted with a cohort of greater than 300 SLE patients compared with healthy controls and patients with other diseases. The data demonstrate that CB-CAPS have diagnostic as well as disease activity monitoring properties that could enhance or even replace current unsatisfactory modalities. Pilot data have also been generated from patients with HCV infection, cardiovascular disease, transplantation, and complications of pregnancy to support the hypothesis that CB-CAPs may serve as universal biomarkers of inflammation.

Epigenetic Biomarkers of SLE

Epigenetic aberrations plays key roles in the etiology of SLE. Interactions between DNA methylation, histone modifications, and miRNAs are factors in pathogenesis of SLE (Zhao et al. 2010). Advances in our knowledge of epigenetics in SLE will lead to discovery of new diagnostic and prognostic biomarkers as well as novel therapeutic targets and strategies.

Levels of methylation of certain genes are closely associated with the onset of SLE. Sensigen has licensed these epigenetic biomarkers to develop an assay, EpiSense™ Lupus, based on its AttoSense technology, which is combined PCR-MS. This is a quantitative methylation-specific assay for the determination of epigenetic changes in SLE-associated biomarkers.

High-resolution analysis of the biological characteristics of plasma DNA in SLE patients using massively parallel genomic and methylomic sequencing has revealed a number of plasma DNA abnormalities (Chan et al. 2014). First, aberrations in measured genomic representations (MGRs) were identified in the plasma DNA of SLE patients; the extent of the aberrations in MGRs correlated with anti-dsDNA antibody level. Second, the plasma DNA of active SLE patients exhibited skewed molecular size-distribution profiles with a significantly increased proportion of short DNA fragments; the extent of plasma DNA shortening in SLE patients correlated with the SLE disease activity index (SLEDAI) and anti-dsDNA antibody level. Third, the plasma DNA of active SLE patients showed decreased methylation densities; the extent of hypomethylation correlated with SLEDAI and anti-dsDNA antibody level. To explore the impact of anti-dsDNA antibody on plasma DNA in SLE, a column-based protein G capture approach was used to fractionate the IgG bound and non-IgG bound DNA in plasma. Compared with healthy individuals, SLE patients had higher concentrations of IgG-bound DNA in plasma. More IgG binding occurs at genomic locations showing increased MGRs. Furthermore, the IgG-bound plasma DNA was shorter in size and more hypomethylated than the non-IgG bound plasma DNA. The shortening of plasma DNA in SLE patients may be a result of the increased production or decreased clearance of short DNA fragments. Hypomethylation of T cells in SLE patients with release of DNA from them might be another mechanism that could contribute toward the hypomethylation of plasma

DNA of SLE patients. These observations have enhanced our understanding of the spectrum of plasma DNA aberrations in SLE and may provide new biomarkers for SLE.

Genetic Loci of SLE

The risk of SLE is influenced by complex genetic and environmental contributions. Alleles of HLA-DRB1, IRF5, and STAT4 are established susceptibility genes; there is strong evidence for the existence of additional risk loci. Two new genetic loci for SLE – a promoter-region allele associated with reduced expression of BLK and increased expression of C8orf13 and variants in the ITGAM-ITGAX region – have been identified and then confirmed through replication (Hom et al. 2008).

HMGB1

Extracellular high-mobility group box 1 (HMGB1), a nuclear DNA-binding protein that functions as an alarmin when released from cells, has been implicated HMGB1 in the pathogenesis of SLE. Elevated concentrations of HMGB1 are observed in sera as well as in skin lesions of patients with SLE andSsrum HMGB1 as well as anti-HMGB1 autoantibody levels correlate with disease activity. HMGB1 is complexed with nucleosomes, at least partially, in the blood of patients with SLE. Moreover, HMGB1-nucleosome complexes from apoptotic cells activate antigen-presenting cells. Injection of HMGB1-nucleosome complexes into nonautoimmune mice results in the formation of autoantibodies against dsDNA and histones in a Toll-like receptor (TLR) 2-dependent manner (Urbonaviciute and Voll 2011). Additionally, HMGB1, as a part of DNA-anti-DNA immune complexes, can interact with receptor for advanced glycation end products (RAGE) on the surface of plasmacytoid dendritic cells and B cells leading to TLR9-dependent IFN-α release and activation of autoreactive B cells, respectively. HMGB1 attached to neutrophil extracellular traps may contribute to IFN-α production by facilitating the recognition of self-nucleic acids. Furthermore, HMGB1, complexed with DNA and pathogenic anti-DNA autoantibodies, activates its receptors, TLR2, TLR4 and RAGE, and may thereby be involved in anti-DNA autoantibody-induced kidney damage in lupus nephritis. These findings suggest that HMGB1 is a potential biomarker of disease activity and, because of its probable involvement in the pathogenesis, a novel therapeutic target in SLE.

Biomarkers of Systemic Sclerosis

Systemic sclerosis is an autoimmune disorder affecting the connective tissue and characterized by thickening of the skin caused by accumulation of collagen as well as injuries to the smallest arteries. It may progress to involve the visceral organs, including the kidneys, heart, lungs and gastrointestinal tract. Plasmacytoid dendritic cells have been implicated in the pathogenesis of systemic sclerosis through mechanisms beyond the previously suggested production of type I interferon.

Proteome-wide analysis and validation has shown that CXCL4 is the predominant protein secreted by plasmacytoid dendritic cells in systemic sclerosis, both in circulation and in skin. A study has found that levels of CXCL4 were elevated in patients with systemic sclerosis and correlated these with the presence and progression of complications, such as lung fibrosis and pulmonary arterial hypertension (van Bon et al. 2014).

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Chapter 8
Biomarkers of Musculoskeletal Disorders

Introduction

Most of the disorders discussed in this section involve bones and joints with a short subsection on muscle disorders. Biomarkers of muscular dystrophy are described in Chap. 14 (neurological disorders). Several methods have been used for detection of biomarkers of musculosketal disorders.

Scientists at Duke University (Durham, NC) have devised a method of determining, in a serum sample, the proportion of the total amount of a biomarker molecule that is derived from catabolism due to the presence of age-related molecular alterations in the molecule. An increased amount of D-aspartate and/or advanced glycation end product in the sample of the subject as compared to the amount of D-aspartate and/or advanced glycation end product in the sample of the control subject is diagnostic of a musculoskeletal, arthritic or joint disorder in the subject and/or identifies a subject at risk of developing such a disorder. A patent has been filed for this invention.

Muscle Disorders

Structure and function of muscles have been studied extensively in health and disease. Biopsies are performed frequently and there is an interest in study of biomarkers as well.

Biomarkers of Muscle Fatigue During Exercise

Biomarkers of peripheral muscle fatigue (BPMFs) are used to offer insights into mechanisms of exhaustion during exercise in order to detect abnormal fatigue or to detect defective metabolic pathways. This aims at describing Advances and future

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perspectives concerning the most important biomarkers of muscle fatigue during exercise have been. Reviewed (Finsterer 2012). BPMFs are classified according to the mechanism of fatigue related to adenosine triphosphate (ATP) metabolism, acidosis, or oxidative metabolism. Muscle fatigue is also related to an immunological response. Impaired calcium handling, disturbances in bioenergetic pathways, and genetic responses. The immunological and genetic response may make the muscle susceptible to fatigue but may not directly cause muscle fatigue. Production of BPMFs is predominantly dependent on the type of exercise. Requirements for BPMFs are: (1) change to reflect the process being monitored; (2) stability without significant diurnal variations; (3) good correlation with exercise intensity; and (4) presence in detectable amounts in easily accessible biological fluids. The most well-known BPMFs are serum lactate and IL-6. The most widely applied clinical application is screening for defective oxidative metabolism in mitochondrial disorders by means of the lactate stress test. The clinical relevance of most other BPMFs, however, has not been validated yet as they often vary according to age, gender, physical fitness, energy supply during exercise, type of exercise needed to produce the BPMF, and whether healthy or diseased subjects are investigated. In spite of these limitations, measuring of BPMFs under specific, standardized conditions appears to be helpful for assessing biological states or processes during exercise and fatigue.

Biomarkers of Mitochondrial Content in Skeletal Muscle

Skeletal muscle mitochondrial content varies widely between human subjects. Biochemical measures of mitochondrial proteins, enzyme activities and lipids are often used as biomarkers of mitochondrial content and muscle oxidative capacity (OXPHOS). A study has investigated the association of these commonly used biochemical measures to to muscle mitochondrial content and OXPHOS in young healthy male subjects (Larsen et al. 2012). A graded exercise test was used to determine maximal oxygen uptake (VO_2 peak) and muscle biopsies were done. Mitochondrial content was determined using transmission electron microscopy imaging. Biomarkers assessed were citrate synthase (CS) activity, cardiolipin content, mtDNA, and complex I–IV activity. Mitochondrial content varied from 4% to 15% of cell volume. Cardiolipin content showed the strongest association with mitochondrial content followed by CS and complex I activities. mtDNA was not related to mitochondrial content. Complex IV activity showed the strongest association with OXPHOS followed by complex II activity. It was concluded that cardiolipin content, and CS as well as complex I activities are the biomarkers that show the strongest association with mitochondrial content, while complex IV activity is strongly associated with OXPHOS capacity in human skeletal muscle.

Idiopathic Inflammatory Myopathies

Idiopathic inflammatory myopathies (IIMs) are rare autoimmune diseases characterized by muscle inflammation, leading to proximal muscle weakness and disability, distinct cutaneous rashes, ulceration, calcinosis, malignancy and interstitial lung disease. Autoimmunity is believed to have a key role in the pathogenesis of myositis and, as such, autoantibodies have been identified in >50% of patients with IIM. It has been demonstrated that these autoantibodies target both nuclear and cytoplasmic components of the cell and they have traditionally been divided into two subsets, myositis-associated autoantibodies (MAAs) and myositis-specific autoantibodies (MSAs). Although diagnostic and classification criteria for myositis include EMG, muscle biopsy and muscle enzymes, inclusion of MSAs/MAAs is limited due to paucity of commercially available assays.

MSA/MAAs can be detected by a variety of methods, with each assay having distinct advantages and disadvantages of sensitivity, specificity, throughput, cost and required expertise. Standard tests include indirect immunofluorescence using HEp2 cells, gel-based techniques of counter-immune electrophoresis, and ELISAs. Several novel myositis autoantibodies including anti-TIF1 (transcription intermediary factor 1), anti-NXP2 (nuclear matrix protein 2), anti-MDA5 (melanoma differentiation-associated gene 5), anti-SAE (small ubiquitin-like modifier activating enzyme;), anti-HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase) and anti-cN1A (cytosolic 5'nucleotidase 1A) have been identified, which correlate with distinct clinical manifestations and are found in inclusion body, statin-induced, clinically amyopathic and juvenile groups of myositis patients. A review has described the main myositis-specific and myositis-associated autoantibodies as well as preliminary studies investigating correlations between specific myositis autoantibody titers and biomarkers of disease course (Betteridge and McHugh 2016). Myositis autoantibodies are useful as both diagnostic and prognostic biomarkers of disease. They can help in predicting further complications of the disease and possible responses to treatment. Further studies are needed to identify novel autoantigenic targets in patients who are currently regarded as autoantibody negative and to understand the role of these autoantibodies and their autoantigens in the pathogenesis of myositis.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a complex inflammatory disease with numerous clinical symptoms and inadequately defined biomarkers. Specific disease, tissue and prognostic biomarker signatures can be identified and characterized without prior selection of clusters of candidate genes directly in the clinic. These strategies not only redefine biomarkers in complex diseases but also reassess our unappreciated disease mechanisms. RA in the most common inflammatory joint disorder.

Tools are now available to evaluate the target tissues in RA proof-of-concept clinical trials. Synovial biomarkers are not yet qualified as surrogate endpoints for regulatory purposes. However, evaluation of intermediate synovial biomarkers provides unique opportunities for guided development and decision-making. Targeted tissue measurements are particularly useful in diseases like RA where patient stratification and target identification are integrated into the early clinical trial process. However, current methods of differentiating RA from other autoimmune disorders such as osteoarthritis and systemic lupus erythematosus have poor specificity and/or sensitivity.

Assays for Biomarkers of RA

The OMERACT 9 Soluble Biomarker Group has successfully formulated a levels of evidence scheme and a study design template that will provide guidance to conduct validation studies in the setting of soluble biomarkers proposed to replace the measurement of damage endpoints in RA, psoriatic arthritis, and ankylosing spondylitis (Maksymowych et al. 2009).

In 2009, the FDA cleared SQiDworks™ Diagnostics Platform (SQI Diagnostics Inc) and its multiplexed IgXPLEX™ RA assay for marketing in the US. The platform incorporates the Company's proprietary IgXPLEX technology to allow multiplexed measurement of target antibody sub-classes (IgA, IgG, IgM) for multiple biomarkers.

NexDx (San Diego, CA) started a collaboration with the University of California, San Diego in 2012 to develop and commercialize a diagnostic test for RA based on epigenetic biomarkers. Discovery of novel DNA methylation biomarkers may be useful in determining whether a patient has RA. Inflammation-producing cells that line the joints of RA patients are characterized by DNA methylation patterns, which produce DNA signatures that could be used as diagnostic biomarkers for RA and identified in patient blood samples. NexDx will also investigate aberrant DNA methylation signatures to determine the optimal therapy and to discover new drug targets for pharma partners.

Biomarkers for Personalizing Therapy of Rheumatoid Arthritis

Vectra™ DA (Crescendo Bioscience), multi-biomarker blood test for RA may more accurately identify RA patients at risk for progression of joint damage when compared to established disease activity measures. Data demonstrate the Vectra DA algorithm score can identify patients at higher risk for structural damage despite achieving remission by DAS28CRP (the 28-joint disease activity score based on C-reactive protein). Among RA patients in DAS28CRP remission, those who also had a high Vectra DA algorithm score are 2.3 times more likely to have progressive

joint destruction during the following year. Moreover, patients in remission as defined by the Vectra DA algorithm score have a lower observed rate of radiographic progression compared to patients in remission by DAS28CRP or by the Boolean criteria of American College of Rheumatology and the European League Against Rheumatism.

Patients with (RA) are generally treated with tumor necrosis factor (TNF)-α inhibitors as second-line therapy if an oral medication such as methotrexate is not adequate to control the symptoms. If one anti-TNF therapy does not lead to adequate symptom control, the current standard of care dictates switching to another approved anti-TNF agent, even though response rates deteriorate with each cycle. Pilot research at Biogen Idec and academic collaborators has developed a panel of gene expression biomarkers with ~90% positive and negative predictive values to identify individuals who did not achieve European League Against Rheumatism (EULAR) Disease Activity Score (DAS)-28 Good response after 14 w of treatment. Such a biomarker panel could be used as a diagnostic test to direct therapeutic options. A clinical study supports the role of the -174G/C IL-6 polymorphism as a genetic marker of responsiveness to anti-TNF therapy according to EULAR criteria (Dávila-Fajardo et al. 2014).

BATTER-UP (Biomarkers of Anti-TNF-α Therapy Efficacy in Rheumatoid arthritis to define Unresponsive Patients) is a clinical study sponsored by Biogen-Idec for adults diagnosed with RA to predict if a specific person with RA will be helped by anti-TNF-α medications such as Remicade®, Enbrel®, Humira®, Cimzia® and Simponi®. The primary outcome measure will be validation of the ability of an 8-gene biomarker set to differentiate between patients who meet or do not meet EULAR DAS-28 Good response criteria after treatment with anti-TNF therapy. The aim is to develop a test that could help physicians decide whether or not to prescribe an anti-TNF drug for a particular patient.

Circulating Cytokines in RA

Circulating cytokines have been demonstrated to correlate with RA disease activity and participate directly in disease pathogenesis. Presence of TNF-α in serum or synovial fluid is a strong indicator of disease activity in RA. TNF-α serves both as a biomarker and a target for therapy. Neutralization of TNF-α with an antibody relieves pain and improves mobility in RA patients. Regulatory T cells are functionally compromised in RA, and indicate that modulation of regulatory T cells by anti-TNF-α therapy may be a further mechanism by which this disease is ameliorated. Etanercept, a TNF-α inhibitor, reduces the oxidative stress biomarker levels (DNA damage, lipid peroxidation, and protein glycosylation) in patients with RA (Kageyama et al. 2008). Elevated levels of serum VEGF have been reported in patients with RA. Successful disease therapy results in lowering the levels of VEGF. Serum levels of VEGF have been reduced by administration of a IL-6 antibody with improvement in patients' condition.

Epigenetic Biomarkers of Rheumatoid Arthritis

RA synovial fibroblasts (RASFs) are the effector cells of cartilage and bone destruction, which show an 'intrinsically' activated and aggressive phenotype resulting in the increased production of matrix-degrading enzymes and adhesion molecules. The three main mechanisms of epigenetic control – DNA methylation, histone modifications and miRNA activity – interact in the development of the RASF phenotype. Normal synovial fibroblasts cultured in a hypomethylating milieu acquire an activated phenotype similar to that of RASFs. These findings suggest that epigenetic control, in particular the control of DNA methylation, is deficient in RASFs. Genome-wide analyses of the epigenome will enable the detection of additional genes involved in the pathogenesis of rheumatoid arthritis, the identification of epigenetic biomarkers, and potentially the development of a therapeutic regimen that targets activated RASFs (Karouzakis et al. 2009).

NexDx Inc. has licensed epigenetics discoveries in RA from the University of California, San Diego to discover novel DNA methylation biomarkers, which can be reliably measured in a blood sample and can serve as an objective indicator of whether or not a patient has RA. Using its Methylome Discovery™ computational platform that helps accelerate the discovery of novel biomarkers, NexDx plans to develop and commercialize a diagnostic test for RA based on these biomarkers.

miRNA Biomarkers in RA

miRNAs dysregulated in RA are shown in Table 8.1.

Serum miR-223 is already proposed as a potential biomarker for sepsis and is now shown expressed in higher levels in RA samples than in OA tissues, and inversely correlated with tender joint count. miR-223 is chiefly known for its role in innate immunity and is the only miRNA that is markedly upregulated in peripheral naive CD4+ T-lymphocytes from RA patients compared with healthy donors.

Finally, miR-146a and miR-155 are the most frequently reported miRNAs deregulated in RA samples including blood, plasma, synovial fluid, PBMCs, CD4+ T cells isolated from the blood or the synovial fluid, or RA-FLS. Increased miR-146a expression levels are correlated with active disease in RA patients. They are both involved in the development of innate and adaptive immune cells, and numerous studies report their upregulation in inflammatory conditions.

Serum CRP in RA

C reactive protein (CRP) has been used as a biomarker of chronic inflammation in RA. CRP is increasingly being incorporated into clinical algorithms to compare disease activity between patients and to predict future clinical events. Findings of a

Table 8.1 miRNAs deregulated in rheumatoid arthritis tissues

	miR16	miR124a	miR132	miR146a	miR155	miR203	miR223	miR346	miR363	miR498
Joint										
ST	+		+		+		+			
FLS		−		+	+	+		+		
SF	+		+	+	+					
CD4+				+						
Blood										
PBMC	+		+	+	+				+	
Serum/ Plasma	+		−	+	+		+			
CD4+				+			+		−	−

ST synovial tissue, *FLS* fibroblast-like synoviocytes, *SF* synovial fluid, *CD4+* T cell lymphocytes positive for the CD4 phenotypic marker, and *PBMC* peripheral blood mononuclear cells. (+) increased or (−) decreased expression levels
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study raise questions about the interpretation of acute-phase serum CRP, particularly, failure to take into account the potential of genetic effects that may result in the inappropriate reassurance or suboptimal treatment of patients simply because they carry low CRP-associated genetic variants (Rhodes et al. 2010). For example, where access to effective, but expensive, biological therapies for RA is rationed on the basis of a DAS28-CRP clinical activity score, then two patients with identical underlying disease severity could be given, or denied, treatment on the basis of CRP genotype alone. The accuracy and utility of these algorithms might be improved by using a genetically adjusted CRP measurement.

Biomarkers of Spondylarthritis

Spondylarthritis (SpA) refers to a group of chronic autoimmune arthritic conditions, including ankylosing spondylitis (AS) and the arthritis associated with psoriasis and inflammatory bowel diseases, primarily affecting peripheral joints. Synovial tissue analysis presents an ideal vehicle for identifying biomarkers of SpA and holds the promise to facilitate the progress of clinical trials dedicated to improving the treatment of SpA, e.g. using tumor necrosis factor (TNF-α) blocker – infliximab or etanercept. Among the synovial features linked to anti-TNF-α effectiveness, there are changes in subsets of synovial macrophages, in the levels of polymorphonuclear cells, and in MMP-3 expression. These findings indicate that, for SpA patients, changes in disease activity are accompanied by series of distinct and measurable events in the peripheral joints. The same synovial features show a highly different standardized response means between SpA patients receiving effective treatment and control patients. In validation analyses with independent synovial tissue samples, effective treatment can be correctly predicted in 80% SpA patients. Further studies are needed to confirm the value of changes observed as biomarkers at early time points across different therapeutic regimens and to combine synovial assessment with predictors of response to treatment in SpA.

Immunopathologic studies have suggested that the features of spondylarthropathy are distinctive, supporting a prominent role for innate immune cells, and can be consistently differentiated from rheumatoid arthritis. Successful treatment of spondylarthropathy synovitis resulted in rapid and sustained decrease in infiltration by macrophage populations and neutrophils, and decreased expression of many proinflammatory mediators. Consistent with studies in rheumatoid arthritis, there are significant correlations between the effects of both methotrexate and infliximab on disease activity and macrophages. These observations highlight the possibility that macrophage populations may be a synovial tissue biomarker of therapeutic intervention in spondylarthropathy. Preliminary studies have evaluated advanced genomic and proteomic methods in spondylarthropathy. A surrogate biomarker of arthritis activity in spondylarthropathy could profoundly enhance screening for efficacy and optimization of dose ranges in early-phase randomized clinical trials.

Biomarkers of Axial Spondyloarthritis

Axial spondyloarthritis (axSpA) is a chronic inflammatory disease involving the spine and presenting with back pain with onset at a young age. If it is not diagnosed and treated, it may result in permanent damage and lifelong disability. Early diagnosis has improved due to MRI, but it is costly and not widely available in all countries, which underscores the need for biomarkers. Key molecules in the pathogenesis of the disease and therapeutic targets such as TNF-α, IL-17 and IL-23, have limited association with disease characteristics or disease progression. Biomarkers such as MMP-3, calprotectin, VEGF, or dickkopf-1 do not adequately reflect disease activity, but may predict progression of structural changes in the spine. Monitoring of calprotectin might represent a valuable biomarker of therapeutic response. Currently, HLA-B27 is the best genetic biomarker for making a diagnosis, while CRP currently appears to be the best circulating measure for assessing disease activity, predicting structural progression and therapeutic response (Prajzlerová et al. 2016). Some genetic biomarkers and particularly anti-CD74 antibodies, may become useful for the early diagnosis of axSpA. However, all these biomarkers need to be validated in trials before introduction into daily clinical practice.

Biomarkers of Psoriatic Arthritis

Psoriasis is an immune-mediated skin disease, which affects 2–4% of the worldwide population, and ~20–30% of patients with psoriasis develop psoriatic arthritis (PsA), a frequently disabling condition. Because skin manifestations precede joint symptoms in with PsA, identification of biomarkers for early prediction of joint damage is an important. There is also a need for identification of biomarkers for predicting therapeutic response which varies considerably among patients. Anti-TNF therapy, IL-12/23-targeted therapy, and IL-17A blockade have shown efficacy but some patients remain resistant to the treatment or experience severe side effects. In addition to measuring disease activity, biomarkers may guide therapy and monitor adverse reactions to specific treatments. Several biomarkers under investigation for OsA include (Paek et al. 2015):

Circulating biomarkers relating to inflammation and cartilage or bone metabolism Serum IL-6, a proinflammatory cytokine produced by lymphoid and other cells, is elevated in patients with PsA versus skin disease alone, correlating with number of joints affected. However, this cytokine is not a specific screening tool as it may be upregulated by other inflammatory processes. Rather, a designated panel of soluble biomarkers may best differentiate patients with psoriatic joint involvement from those with only cutaneous lesions. Other biomarkers are osteoprotegerin, high-sensitivity CRP (hs-CRP), cartilage oligomeric matrix protein (COMP), MMP-3, and VEGF in patients with PsA versus psoriasis alone.

Genetic biomarkers for PsA Genetic factors play a significant role in psoriasis, as initially reported by the identification of psoriasis susceptibility-1 (PSORS1) in family studies and association with major histo-compatibility complex (MHC) haplotypes in case-control studies. Several SNPs are linked with therapeutic efficacy and may serve as biomarkers to predict treatment response but the predictive potential for individual genes in psoriatic disease is minimal.

MicroRNAs Studies investigating the circulating miRNA profiles of patients with psoriatic disease have found significant changes in the expression of several miRNAs when compared with healthy controls. Candidate biomarkers for psoriasis include the upregulated expression of miR-33, miR-369-3p, miR-1266, and miR-128 as well as the downregulation of let-7d, miR-142-3p, and miR-181a.

Osteoarthritis

Osteoarthritis (OA) is a painful disorder of joints characterized by destruction of articular cartilage and remodeling of bone. It is a chronic disease with a long silent period. The diagnosis is generally based on clinical symptoms radiological studies, imaging of the involved joints and arthroscopy. X-ray has a poor sensitivity and a relatively large precision error that does not allow an early detection of OA or the monitoring of joint damage progression. The limitations of the tools that are currently available for OA assessment have been the impetus to identify specific bimarkers that reflect quantitative and dynamic variations in joint remodeling. An understanding of the molecular pathophysiology of OA is an important basis for study of biomarkers of this disease. Research has focused on the structural components of cartilage matrix, especially type II collagen degradation markers.

Molecular Pathophysiology of OA

At the molecular level, loss of the cartilage proteoglycan is clearly evident at a relatively early stage followed by erosion and degradation of other cartilage components (collagen) until the underlying bone is denuded of cartilage. There are some features in common with RA. The breakdown of the cartilage is thought to be mediated by the release of proteolytic enzymes such as matrix metalloproteinases (MMPs). Nitric oxide (NO) plays a role in the pathogenesis of OA. The activation of synovial cells and chondrocytes in OA involves different phases – an early, altered biomechanical and a chronic cytokine-driven auto-destruction, e.g. IL (interleukin)-1. There is a very modest synovial inflammation in OA (compared to RA), with increased MMP (metalloprotease)-1, and MMP-3, but no cytokine overproduction. In the OA cartilage, however, alterations are much more obvious – increased IL-1, TNF-α, and iNOS (induced nitric oxide synthase). The multiple

effects of NO on chondrocytes include increased IL-1, MMPs, apoptosis, proteoglycan, and type II collagen. The production of NO and PGE2 in OA cartilage appears to be responsible for altered biomechanics accompanied by the local increase in inflammatory cyto. Kines (particularly IL-1, but also IL-6 and IL-8) and the differential expression of extracellular matrix elements. IL-1 induces a catabolic phenotype in chondrocytes and increased apoptosis. Antagonizing IL-1-beta but not TNF-α, decreases NO and PGE2 production by OA cartilage explants. NO dependence has been demonstrated in the increased susceptibility to oxidant injury and in the oxidant stress-induced apoptosis in cytokine-stimulated chondrocytes. Furthermore, IL-1 induces peroxynitrite and superoxide anion in chondrocytes. The exposure of chondrocytes to peroxynitrite results in Jun N-terminal kinase activation and translocation of NF-kB to the nucleus. MMP-1 and MMP-9 are regulated in OA cartilage through the fibronectin receptor α3β1 integrin. One of the functions of intraarticular osteopontin (a major non-collagenous proteins of bone matrix), which is overexpressed in OA cartilage, is to act as an innate inhibitor of IL-1, NO, and PGE2 production. This function is regulated by the interaction of chondrocytes with differentially expressed proteins within the extracellular matrix.

Biomarkers of Osteoarthritis

Because of lack of effective methods for early detection and evaluation of treatment outcome in OA, measurement of biomarkers is needed for disease monitoring. Biomarkers OA biomarkers can be easily isolated from biological fluids such as blood, urine and synovial fluid. Several studies deal with identification and development of new biomarkers for OA. A review of these studies has suggested classification of OA biomarkers according to metabolism of involved tissues (bone, cartilage and synovial membrane), inflammatory pathways and biological function (Nguyen et al. 2017). A classification of inflammatory biomarkers of osteoarthritis is shown in Table 8.2.

Table 8.2 Classification of inflammatory biomarkers in osteoarthritis

Tissue of origin	Category	Biomarkers
Cartilage and bone of joints	Cytokines and chemokines	TNF-α, IL-2, IL-4, IL-6, IL-8, IL-15, IL-18
	Growth factors	VEGF
	Lipid mediators	Prostaglandin E2
Liver	Acute phase protein	C-reactive protein
Adipose tissue	Obesity-related inflammatory factors	Adioponectin, resistin, leptin, ApoA1
Macrophages	Cytokines	CCL2, CCL3, CCL4
Neutrophils	Enzyme	Myeloperoxidase.

Assays for Biomarkers of OA

Several assays for biomarkers of OA are commercially available. IBEX Technologies Inc. provides ELISA assays that enable the study of both the synthesis and degradation of connective tissue components in OA.

- Cartilage degradation assays: (1) C2C serum assay measures type II collagen destruction using a MAb to type II collagen primary cleavage; and (2) C1-2C assay measures type I and type II collagen destruction using a specific polyclonal antibody.
- Collagen synthesis assays: (1) The CPII assay (Procollagen II C-Propeptide) measures type II collagen synthesis with an increased level of CPII in serum in RA in contrast to OA with decreased level in serum and an increased level in synovial fluid; and (2) CS-846 (aggrecan chondroitin sulfate 846 epitope) measures newly formed aggrecan with specific IgM MAb that recognizes an epitope present in fetal aggrecan and large aggrecan produced to repair cartilage damage when tissue is affected by RA or OA.
- C2C Human Urine Sandwich Assay measures the progression of collagen II-degradation in OA and RA.

Biomarkers of OA

Efforts are being made to develop a blood-based approach to identify OA of the knee. Blood samples of subjects with arthroscopically diagnosed mild OA of the knee have been screened for differentially expressed genes following RNA isolation and then tested using real-time RT-PCR. Logistic regression analysis was used to evaluate linear combinations of the biomarkers and receiver operating characteristic curve analysis was used to assess the discriminatory power of the combinations. Genes differentially expressed between mild knee OA and control samples are identified by microarray analysis. Subsequent real-time RT-PCR verification has shown that genes significantly down-regulated in mild OA include: HSP 90 kDa, α1; inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein; IL-13 receptor α1; laminin, γ1; platelet factor 4 (also known as chemokine (C-X-C motif) ligand 4) and TNF, alpha-induced protein 6. Logistic regression analysis has identified linear combinations of genes that can discriminate between subjects with mild OA and controls. Linear combinations of blood RNA biomarkers offer a substantial improvement over currently available diagnostic tools for mild OA. Blood-derived RNA biomarkers may be of significant clinical value for the diagnosis of early, asymptomatic OA of the knee.

The National Institute of Arthritis and Musculoskeletal and Skin launched the Osteoarthritis Initiative, a public-private partnership between the NIH and industry that funds a multisite contract to create a resource to hasten discovery of biological markers for OA. Men and women age 45 and older at risk for developing OA and

those with early disease were eligible to participate. After an initial screening, four centers around the US each enrolled and followed 1250 adults for 5 years (total enrollment of 5000). Biological specimens (blood, urine, DNA), images (X-rays and MRI scans) and clinical data were collected annually. Regional analysis of femorotibial (FT) cartilage loss in a subsample from the Osteoarthritis Initiative progression subcohort showed that the rate of cartilage loss is greater in central subregions than in entire FT cartilage plates (Wirth et al. 2009).

Concluding Remarks and Future Prospects of Biomarkers of OA

The Osteoarthritis Research Society International (OARSI), an organization devoted to the study of OA, has established an OA Biomarkers Global Initiative. With support from the National Institute of Arthritis, Musculoskeletal and Skin Diseases of NIH and the Arthritis Foundation, OARSI has developed a series of workshops since 2009 that have brought together scientists from around the world for meetings. The broad goals for OA research are aimed at: developing a paradigm of molecular, pre-radiographic, and radiographic OA that can be used in clinical trials; identifying subgroups such as early OA and post-injury OA; influencing research to advance biomarker development; optimizing use of existing samples and clinical study resources; and developing a study of current biomarkers using the samples from the Osteoarthritis Initiative of the US Government (Kraus et al. 2010). The goal of current research is to move the diagnosis of OA from a radiologic viewpoint back to a pre-radiologic viewpoint and on to the molecular events that initiate cartilage breakdown and joint failure (Sandell 2012).

In spite of a significant increase of some biomarkers in individuals with early stage of OA, the large overlap with control subjects indicates that the current biomarkers used alone have limited diagnostic potential. However, the combination of specific biomarkers seems to improve the prediction of disease progression at the individual level. Several types of treatment have been investigated but the lack of drugs with definitive chondroprotective activity has limited the assessment of the potential role of biomarkers for monitoring patients' responses to the treatment of OA (Rousseau and Garnero 2012).

Biomarkers of Osteoporosis

Osteoporosis is a progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. Osteoporosis is an important public health concern in older women. More than one-third of women will suffer one or

more osteoporotic fractures in their life time. Postmenopausal women are at greater risk of osteoporosis; however, not all women will have the same risk of developing osteoporosis. Life time risk among men is less but still substantial.

Assays for Detection of Biomarkers of Osteoporosis

Type I collagen molecules in bone matrix are linked together by cross-linking molecules including pyridinoline (PYD) and deoxypyridinoline (DPD) in the region of N and C-telopeptides. DPD, which differs from PYD by the absence of an hydroxyl residue, is specific for bone tissue. During bone resorption, pyridinoline is released into the circulation and excreted in the urine in its free form or linked to C- (CTX) or N- (NTX) telopeptides. The different cross-link forms can be measured in serum and urine using specific immunoassays. A targeted use of biomarkers could optimize identification of high-risk patients, the process of drug discovery, and monitoring of the efficacy of osteoporosis treatment in clinical settings.

Roche's Elecsys® β-CrossLaps bone resorption biomarker is an immunoassay used to determine the amount of degradation products, i.e., CTX as an aid in assessing bone resorption (Fig. 8.1). The test may be used as an aid in monitoring antiresorptive therapies in postmenopausal women and patients diagnosed with osteopenia.

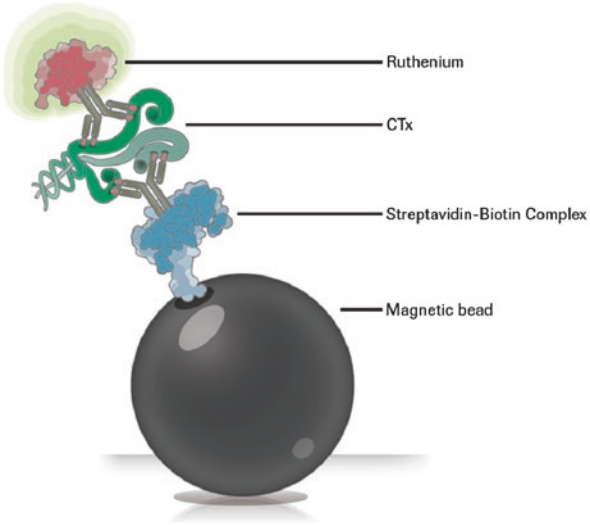

Fig. 8.1 β-CrossLaps bone resorption biomarker assay. Legend for Fig. 8.1: β-CrossLaps is an electrochemiluminescence immunoassay that uses a sandwich complex, which is made from a two monoclonal anti-β-CrossLaps antibodies – one is biotinylated and one is labeled with a ruthenium complex. These two antibodies bind to the antigen and then interact with streptavidin-coated microparticles which are magnetically captured by an electrode. When a voltage is applied to the electrode it induces a chemiluminescent emission which is measured. The amount of antigen present correlates with the amount of emission measured (Roche Diagnostics Corporation, reproduced by permission)

Bone Imaging with Quantitative CT and MRI

Quantitative CT (QCT) and MRI enable independent measurements of trabecular and cortical BMD as well as bone structure and derived measures of bone strength. QCT and MRI produce tomographic images of the bone which provide 3D images of bone geometry which can be used to measure cortical bone dimensions.

Circulating miRNAs as Biomarkers of Osteoporosis

Circulating miRNAs are remarkably stable analytes that can be measured using qPCR, but their analysis faces several pre-analytical as well as analytical challenges. There is accumulating evidence that miRNAs play an essential role in the regulation of bone homeostasis, and specific changes in miRNA transcription levels or miRNA secretory levels have been linked to the development and progression of certain bone diseases. Results from circulating miRNAs analysis in patients with osteopenia, osteoporosis and fragility fractures have been reported. By comparing these findings to studies on circulating miRNAs in cellular senescence and aging or muscle physiology and sarcopenia, several overlaps were observed. This suggests that signatures observed during osteoporosis might not be specific to the pathophysiology in bone, but rather integrate information from several tissue types (Hackl et al. 2016). Further work is in progress to validate circulating miRNAs as robust diagnostic tools for bone diseases in clinical research, clinical routine and in personalized medicine. Another study in postmenopausal women found that miR-382-3p significantly enhanced osteogenic differentiation, while miR-550a-5p inhibited this process; both impaired adipogenic differentiation, while miR-188-3p did not exert an effect on adipogenesis (Heilmeier et al. 2016). None of the miRNAs affected cell proliferation significantly. These data suggest for the first time that miRNAs are linked to fragility fractures in type 2 diabetic postmenopausal women and should be further investigated for their diagnostic potential and their detailed function in diabetic bone. osteomiR™ (TAmiRNA GmbH) is a signature of serum miRNAs that is associated with bone quality and is intended to assess the risk of a first fracture in postmenopausal and type-2 diabetic women.

Dual X-ray Absorptiometry

Dual x-ray absorptiometry (DXA) scanners are widely available and offer an inexpensive and precise method to assess bone mineral density at many skeletal sites including the lumbar spine, proximal femur, forearm and total body. However the quality of DXA measurements varies widely from clinic to clinic and among geographical regions. In addition, the performance of the scanner itself impacts the

quality of the measurement. When adjusting for differences in calibration between manufacturers, better results may be achieved by using standardization equations developed from scans of human beings. The DXA manufacturers have adopted formal, generalized standardization equations developed through the activities of the International Committee for Standards in Bone Measurement. DXA combined with molecular biomarkers of bone degradation is more predictive of fracture risk than either DXA or biomarkers alone.

Utility of Biomarkers of Osteoporosis

Bone mineral density (BMD) and molecular biomarkers of bone turnover are useful endpoints in phase II dose-ranging trials and have the advantage in that they enable shorter trials with fewer subjects. There is no linear relation between changes in BMD and reduction in fracture risk with antiresorptive agents. Interpretation of BMD changes at the individual level requires calculating the smallest significant change at each measurement center. BMD measurement is essential before administration of antiresorptive or anabolic agents for prevention or treatment of postmenopausal osteoporosis. Biomarkers of bone turnover can be monitored after a few months of treatment but their interpretation requires careful assessment of their intraindividual variability. The validity of bone mineral density and biomolecular markers as surrogates for fracture in clinical trials is not yet established. Thus therapeutic confirmatory studies require fracture as an endpoint. Various fracture endpoints have been employed including morphometric vertebral fracture, hip fracture and all clinical fractures.

In clinical practice, measurement of bone turnover biomarkers is useful for investigation of patients with osteoporosis. Among the available biochemical markers, the measurements of serum procollagen type I N-terminal propeptide (PINP) and the crosslinked C-terminal telopeptide (serum CTX) have been recommended as reference markers of bone formation and bone resorption, respectively (Garnero 2017). The important sources of preanalytical and analytical variability have been identified for both biomarkers, and precise measurement can now be obtained. Protein-based biomarkers have some limitations, including a lack of specificity for bone tissue, and their inability to reflect osteocyte activity or periosteal metabolism. Therefore, novel biomarkers such as periostin, sclerostin and, sphingosine 1-phosphate have been developed to address some of these shortcomings. Measurements of circulating miRNAs may represent early biomarkers in osteoporosis. Biomarkers of osteoporosis are a useful adjunct to measurement of bone mineral density for identifying postmenopausal women at high risk for fracture. Because levels of these biomarkers respond rapidly to both anabolic and anticatabolic drugs, they are very useful for investigating the mechanism of action of new therapies and for predicting their efficacy for reducing risk of fractures.

Biomarkers of Osteonecrosis

Osteonecrosis in Gaucher's Disease

Osteonecrosis is a disabling complication of Gaucher disease. Biomarkers that can predict osteonecrosis and monitor the effectiveness of therapies would improve clinical practice and facilitate the molecular exploration of this disorder. Several biomarker molecules, which may ultimately improve risk assessment for osteonecrosis, have been identified. Discovery of prospective biomarkers such as CCL18/PARC, CXCL8/IL-8, CCL5/RANTES, CCL3/MIP-1α, CCL4/MIP-1β, particularly during recurrent episodes despite enzyme treatment, has the potential to radically change the management of Gaucher disease and should improve therapeutic monitoring and prognostic evaluation (Pavlova et al. 2012).

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Chapter 9
Biomarkers of Infectious Diseases

Introduction

Infection is defined as a pathologic process caused by the invasion of normally sterile tissue or fluid or body cavity by pathogenic or potentially pathogenic microorganisms. Sepsis is defined as the presence of organ dysfunction occurring as the result of a dysregulated host response to an infection.

Sepsis

In a young patient with an obvious meningococcal rash, high fever, and altered mental status, diagnosis of sepsis is fairly straightforward, but this is not always the case, especially amongst the critically ill population with multiple comorbidities and other ongoing disease processes. The first clinical sign of sepsis, fever, is usually are not typical or specific. Similarly leukocytosis is nonspecific. The more typical signs or laboratory parameters such as arterial hypotension or lactate accumulation are often are late symptoms associated with organ dysfunction and a rising mortality rate. Traditional laboratory methods for confirming diagnosis of infectious diseases involve microbial identification that relies on morphological features, growth characteristics, and biochemical substrates. Microbiologists have searched for more rapid and efficient means of microbial identification. Nucleic acid amplification technology, PCR, has opened up new frontiers for microbial identification. Advent of molecular diagnostics has provided a tool for faster diagnosis of infections. Even PCR-based methods have time limitation as they cannot be performed within half an hour required for the POC diagnosis of infections. Non-PCR methods have been developed for this purpose and the role of biomarkers that can be detected more

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rapidly is being explored. The diagnosis of infections will, however, continue to require a critical clinical awareness, careful patient history, dedicated physical examination, and appropriate cultures.

The diagnostic spectrum of the various markers, however, is different. Many biomarkers have been implicated as playing a harmful mediator role in sepsis. Some primarily indicate severity of inflammation (e.g. IL-6), others respond to infection, but do not indicate the host response well (endotoxin, lipoprotein binding protein, triggering receptor on myeloid cells). Recent data and cumulative analyses indicate that biomarkers of sepsis improve diagnosis of sepsis, but only a few biomarkers have impact on therapy and fulfill the clinical requirements. Characteristics of an ideal biomarker of infection are:

- High levels in sepsis
- Positive correlation with severity of infection
- Prolonged persistence in the blood
- Should enable an early diagnosis by rapid and accurate bedside measurement
- Should indicate the course and prognosis of the disease
- Should facilitate therapeutic decisions

Several studies indicate that the prohormone procalcitonin, a biomarker-mediator of sepsis, possesses great potential for meeting all of the above criteria, and that its therapeutic immunoneutralization in humans merits evaluation.

Application of Proteomics for Discovering Biomarkers of Infections

Proteomic technologies have been used for detection of protein biomarkers of infection. For example, a novel mass spectral fingerprinting and proteomics approach using MALDI-TOF MS was applied to detect and identify protein biomarkers of group A Streptococcus (GAS) strains (Moura et al. 2008). Specific biomarkers were found for each strain, and invasive GAS isolates could be differentiated. GAS isolates from cases of necrotizing fasciitis were clustered together and were distinct from isolates associated with noninvasive infections.

Endothelial cells play a key role in the inflammatory response triggered by sepsis. Proteomic technologies have been used to investigate the secretome of EA.hy926 endothelial cells following lipopolysaccharide stimulation (LPS). Secretome dynamics in response to LPS were analyzed with an online 2D–LC-MS/MS system (Kwon et al. 2015). Out of the 19 candidate proteins, the authors focused on moesin (membrane-organizing extension spike protein), which is involved in the function of endothelial cells, and confirmed its amount in cellular lysates and media taken from primary human umbilical vein endothelial cells treated with LPS. The findings indicate that moesin is a potential biomarker of sepsis.

Biomarkers of Sepsis

There are difficulties in diagnosing sepsis from organ dysfunction. The availability of accurate sepsis biomarkers to facilitate diagnosis could be useful to enable timely appropriate treatment to be started, thus optimizing a patient's chances of survival. More than 170 biomarkers have been proposed and assessed clinically, including various cytokines, cell surface biomarkers, receptors, complement factors, coagulation factors, acute phase reactants, and many others, but none has 100% specificity for sepsis. Perhaps the most widely studied biomarker of sepsis is CRP, whose role in host defense against bacteria has been known about for almost 100 years. However, CRP is sensitive but not very specific, as it is increased in all inflammatory disorders, including after uncomplicated surgery. PCT, first proposed as a biomarker in 1993, is perhaps a more specific biomarker than CRP, although it is also increased in other inflammatory conditions, such as pancreatitis or after polytrauma or major surgery.

Biomarkers play a role in helping to identify—or perhaps more importantly rule out—an infection. Infection is not an all-or-none phenomenon, and there are "gray areas" where one can never really be certain that an infection was present or absent. Because of their high sensitivity, sepsis markers are usually more helpful at ruling out than at ruling in an infection. This is particularly true in critically ill patients, who often have some inflammatory response, but do not always have infection or require antibiotic administration. Hence, sepsis biomarkers, by ruling out infection, could help decrease the use of unnecessary antibiotics, limit the use of excessive imaging procedures in search of a possible source, and encourage the clinician to search for alternative diagnoses. One example of this use for biomarkers was use of PCT to rule out infections in febrile patients presenting to an emergency department. In patients with suspected lower respiratory tract infection, use of antibiotics was more or less discouraged based on the PCT concentration. The result was a significant reduction in antibiotic use.

The second question relates to their role in assessing the severity of disease, primarily for triaging decisions, eg, whether or not to admit a patient from the emergency room or general ward to the ICU. An example of this use for biomarkers was demonstrated by in 1156 hospitalized patients, showing that mortality rates were 2.6 times higher in patients presenting with sepsis on the general ward with a PCT concentration > 0.12 ng/mL than in those with lower PCT levels (Giamarellos-Bourboulis et al. 2011). The authors suggested that PCT concentrations could thus be used to help identify which patients may benefit from ICU admission.

The third question relates to their role in monitoring a patient's response to therapy. For this role in particular, trends in concentrations over time are clearly of more value than single measurements. Again, PCT and CRP are the most widely studied biomarkers in this context. The mortality rates are substantially lower in septic patients in whom the PCT concentration decreased by more than 50% over 72 h than in the other patients (Karlsson et al. 2010). Similarly, the pattern of change in CRP concentrations correlated with the individual clinical course in

Table 9.1 Biomarkers of sepsis

Carbamoyl phosphate synthase-1 (CPS-1)
Chemokines
Coagulation system markers
C-reactive protein
CoQ10 level reduction
Endotoxin
Inducible nitric oxide synthase (iNOS)
Lactate
Leukocytosis
Lipoprotein Binding Protein
Moesin
Pro-atrial natriuretic peptide
Procalcitonin
Proinflammatory cytokines
soluble urokinase Plasminogen Activator Receptor (suPAR)
Triggering receptor on myeloid cells

patients with community-acquired sepsis (Povoa et al. 2011). A progressive decline in CRP or PCT concentrations can be used to guide earlier discontinuation of antibiotic therapy, without major risks. But an increase in CRP concentrations in the first 48 h of therapy suggests that antibiotic therapy may be ineffective and need reevaluation. PCT levels have been used to guide antibiotic therapy in several clinical trials in different groups of infected patients with promising results on antibiotic use. However, using PCT concentrations in an antibiotic-escalation strategy is not a wise strategy, as it may result in worse outcomes. Clearly, clinical decisions should not be based just on the concentrations of a single biomarker but must include evaluation of the clinical status of the patient and other hemodynamic and laboratory parameters. Several biomarkers of sepsis are currently available as listed in Table 9.1.

Circulating CPS-1 as Biomarkers of Organ Damage in Sepsis

Mitochondrial damage and dysfunction are considered to play an important role in the pathogenesis of sepsis-induced organ failures. Unfortunately, there is paucity of specific biomarkers of mitochondrial damage in vital organs. Carbomyl phosphate synthase (CPS)-1, a protein primarily localized to liver mitochondria, is present in high concentrations in the plasma of patients with sepsis. A prospective, randomized, controlled animal study has verified that circulating CPS-1 is a biomarker of mitochondrial damage and depletion in the liver during the subacute phase of sepsis (Crouser et al. 2006). From a mechanistic standpoint,

mitochondrial depletion is not due to cell death but is apparently related to the removal of damaged mitochondria by lysosomes, followed by repletion of mitochondrial populations. Restoration of mitochondrial populations in the liver and reduced levels of CPS-1 appears to signal recovery from sepsis. CPS-1 may be superior to conventional biomarkers of liver damage during sepsis. Further studies are needed to determine the clinical utility of CPS-1 as a biomarker of severity of sepsis.

CoQ10 Level Reduction in Septic Shock

The relationship between CoQ10 levels and inflammatory and vascular endothelial biomarkers was assessed using Pearson or Spearman correlations during analysis of a prospective randomized trial of simvastatin versus placebo in patients with septic shock (Donnino et al. 2011). CoQ10 levels were significantly decreased in patients with septic shock compared to healthy controls. CoQ10 is inversely associated with vascular endothelial biomarkers and inflammatory molecules though this association diminishes when adjusting for levels of low density lipoprotein (LDL) cholesterol, which is the primary transport molecule for CoQ10. Only vascular cell adhesion molecule (VCAM) and IL-10 remained lower following the adjusted analysis. Identifying low CoQ10 levels in septic shock is significant as the compound is essential to mitochondrial function and may play an important role in the pathophysiology of mitochondrial dysfunction in sepsis. It opens the possibility for potential therapeutic intervention as CoQ10 can be administered exogenously.

Multibiomarker-based Outcome Risk Stratification of Septic Shock

Failure of clinical trials in septic shock is partly due to inequitable and unknown distribution of baseline mortality risk between study arms. Interventional trials in septic shock require effective outcome risk stratification. Genome-wide expression studies have identified 12 plasma proteins as candidates for biomarker-based risk stratification. A multibiomarker-based outcome risk stratification model for adult septic shock, which included five candidate biomarkers, admission lactate concentration, age, and chronic disease burden, had a sensitivity for mortality of 94%, specificity of 56%), positive predictive value of 50%, and negative predictive value of 95% (Wong et al. 2014). The calibrated decision tree had the following test characteristics in the validation cohort: sensitivity 85%, specificity 60%, positive predictive value 61%, and negative predictive value 85%.

Nitric Oxide as a Biomarker of Sepsis

There is a wealth of data implicating nitric oxide (NO) as a key player in all cardiac, vascular, renal and pulmonary derangements of sepsis and septic shock. Cytokines can activate inducible nitric oxide synthase (iNOS) expression, which produces excessive amounts of NO, which can cause shock in sepsis. NO and cytokines constitute the molecular biomarkers and the intercellular messengers of inflammation and septic shock.

Septic shock occurs with an exacerbated inflammatory response that damages tissue mitochondria. Skeletal muscle appears as one of the main target organs in septic shock, showing an increased NO production, an early oxidative stress, and contractile failure. Mitochondria isolated from rat and human skeletal muscle in septic shock show a markedly increased NO generation and a decreased state 3 respiration, more marked with nicotinamide adenine dinucleotide (NAD)-linked substrates than with succinate, without uncoupling or impairment of phosphorylation. One of the current hypotheses for the molecular mechanisms of septic shock is that the enhanced NO production by mitochondrial NOS leads to excessive peroxynitrite production and protein nitration in the mitochondrial matrix, to mitochondrial dysfunction and to contractile failure.

Research & Diagnostic Antibodies (www.rdantibodies.com/) has conducted clinical studies involving >290 ICU patients using tests based on the anti-iNOS MAbs. In 2010, the company received a $2.6 million grant from the National Institute of General Medical Sciences to support a pivotal clinical study of the test designed to obtain FDA clearance. The test, based on a novel plasma biomarker discovered by the company, can identify patients who will develop the sepsis pathology 24 to 48 h prior to the appearance of symptoms currently used by physicians as indictors of the onset of sepsis and enable them to treat patients more effectively by starting antibiotic treatment and fluid resuscitation sooner. The test has not yet been approved by the regulatory authorities.

SuPAR as a Biomarker of Sepsis

A prospective cohort study has evaluated the soluble form of urokinase-type plasminogen activator (suPAR) as an early prognostic biomarker of sepsis in patients with suspected infection (Uusitalo-Seppälä et al. 2012). suPAR was measured on admission using a commercial solid-phase ELISA. At a cut-off level of 6.4 ng mL(−1), suPAR had 76% sensitivity and 69% specificity for fatal disease; at a cut-off level of 6.6 ng mL(−1), the sensitivity and specificity for severe sepsis were 67% and 72%, respectively. The levels were significantly higher in nonsurvivors compared with survivors and in patients with severe sepsis compared with those in the other groups. High suPAR is an independent predictor of case fatality in severe inflammatory response syndrome (SIRS).

Chemokines as Biomarkers of Infection

Chemokines are a superfamily of small peptides involved in leukocyte chemotaxis and in the induction of cytokines in a wide range of infectious diseases. These peptides are secreted by tissue cells, leucocytes and activated epithelial cells. Four different subfamilies can be identified based on the highly conserved presence of the first two cysteine residues, which are either separated or not by other amino acids: the CC chemokines, the CXC chemokines, the CX3C chemokines and the C chemokines. Chemokines act through a family of chemokine receptors, which are present on cell types such as leukocytes, dendritic cells and endothelial cells. Chemokines and their receptors play an important role in the innate immunity against infectious diseases such as HIV/AIDS and malaria. Measurement of the serum levels of CXC and CC chemokines during the initial phase of meningococcal sepsis in children can predict mortality and can correlate strongly with disease severity. Chemokines may play a key role in the pathophysiology of meningococcal disease and are potentially new targets for therapeutic approaches.

Endotoxin as Biomarker of Infection

Endotoxin has been a candidate as a diagnostic tool for infection for several years. However, inconsistently increased levels, variations in sensitivity and specificity in different patients groups and lack of correlation with severity of inflammation and the host response did not support clinical use. It has been reevaluated now by a highly sensitive biological assay, which has been approved by the FDA for use in the US. The endotoxin activity assay (EAA™, Spectral Diagnostics Inc) is quite sensitive and is based on an ex-vivo whole blood measurement system. It measures the zymosan- and anti-endotoxin-antibody elicited respiratory burst in a kinetic luminometric assay. The antibody is directed against lipopolysaccharides of various gram-negative bacteria. Despite being a good biomarker for the exclusion of infection, it does not indicate well the host response. The indication for clinical use of this test thus is limited to the exclusion of infection in patients admitted to ICU. Because of the low response to severity of infection, it may have a limited value as guide to therapy. Also the low specificity may restrict clinical use. Future studies will indicate, whether other biomarkers have a similar sensitivity at a given low specificity.

Procalcitonin as a Guide to Antibiotic Therapy in Infections

Procalcitonin (ProCT), a precursor peptide from the hormone calcitonin (CT), better fulfills the requirements of a desirable biomarker as compared to others and has a solid scientific basis. After translation from CT-mRNA, ProCT is cleaved

enzymatically into smaller peptides, finally to yield the 32 amino acid mature CT. Most CT precursor peptides, including ProCT, are found in the serum of normal persons. In microbial infections and in various forms of inflammation, circulating levels of several calcitonin precursors, including ProCT but not mature CT, increase up to several thousand-fold. This increase and especially the course correlate with the severity of the condition and with mortality. A microbial infection induces an increase of CALC-I gene-expression and release of ProCT from all parenchymal tissues and differentiated cell types throughout the body.

A commercially available assay is based on time-resolved amplified cryptate emission technology (Kryptor® PCT, Brahms/ThermoFisher Scientific). It used a sheep polyclonal anti-calcitonin antibody and a monoclonal anti-katacalcin antibody, which bind to the calcitonin and katacalcin sequence of calcitonin precursor molecules. Diagnostic accuracy of ProCT has been shown for a variety of infections, e.g. respiratory tract infections, meningitis, acute infectious endocarditis and pancreatitis. A colorimetric, "quick" bedside version of the test (PCT®-Q) has the advantage of rapid determination of circulating CTpr levels in 30 min but he assay is only semi-quantitative and is not sensitive enough to detect moderately elevated ProCT levels.

A ProCT-based therapeutic strategy can safely and markedly reduce antibiotic usage in lower respiratory tract infections, the major cause of sepsis. Being a hormokine mediator, immunoneutralization of ProCT might offer new hope for more effective treatment options in sepsis. It is now evidence that ProCT provides more information and, thereby, questions the currently used "gold standards" for the diagnosis of clinically relevant bacterial infections. Yet, ProCT is less than a perfect biomarker. ProCT can be increased in noninfectious conditions, and may remain low in infections.

A randomized intervention trial, conducted on patients with suspected community-acquired pneumonia at the University Hospital (Basel, Switzerland), assessed ProCT guidance for the initiation and duration of antibiotic therapy (Christ-Crain et al. 2006). The primary endpoint was antibiotic use; secondary endpoints were measures of clinical, laboratory, and radiographic outcome. ProCT guidance reduced total antibiotic exposure, antibiotic prescriptions on admission, and antibiotic treatment duration compared with patients treated according to guidelines. Measurements of ProCT, reduced the length of antibiotic treatment by an average of 7 days. ProCT appears to be a more reliable measure for individual tailoring and early discontinuance of antibiotic therapy as compared with the routinely used clinical and other parameters. It is also time- and cost-effective. It took less than 20 min to detect levels of serum procalcitonin in the laboratory and results were routinely available within an hour. Each test costs $15–$30.

Prognosis of patients with severe sepsis and septic shock admitted to the intensive care unit (ICU) may be associated with ProCT. Results of a prospective analysis of patients with sepsis admitted to the ICU indicate that dynamic changes of PCT reflected on day 3 and day 5 after admission to the ICU may serve as a predictor of survival in critically ill patients with severe sepsis (Huang et al. 2016).

Soluble Urokinase Plasminogen Activator Receptor

The soluble urokinase Plasminogen Activator Receptor (suPAR) is a protein in the blood. It is measured by suPARnostic® ELISA assay (ViroGates A/S), a CE/IVD marked double MAb sandwich assay in which samples and peroxidase-conjugated anti-suPAR are first mixed together and then incubated in anti-suPAR precoated microwells. The recombinant suPAR standards of the kit are calibrated against healthy human blood donor samples and suPAR concentrations are given as ng/mL plasma. A prospective cohort study showed that plasma suPAR level is a sensitive and specific independent prognostic biomarker in patients with bacteremia (Huttunen et al. 2011). If an individual's suPARnostic® level is very high, there is an increased chance of negative outcome in critical conditions such as septicemia unless appropriate treatment is administered early, which can lower suPARnostic® level. Thus, by measuring an individual's suPARnostic® level, the prognosis can be assessed, the need for therapy is indicated and the effect of treatment can be monitored.

Systemic Inflammatory Response Syndrome

Sepsis is now defined as a systemic inflammatory response syndrome (SIRS) in which there is an identifiable focus of infection. During the onset of sepsis, a massive inflammatory reaction involving chemical mediators such as cytokines and chemokines and inflammatory cells such as the polymorphonuclear neutrophil and macrophage takes place. In addition to this systemic inflammatory process, sepsis and septic shocks cause a profound decrease in the peripheral vasomotor tone leading to a great decrease in the peripheral resistance. This event is central to derangement of hemodynamic and perfusion parameters. SIRS can be also precipitated by non-infective events such as trauma, pancreatitis, and surgery. As a consequence of an overactive SIRS response, the function of various organ systems may be compromised, resulting in multiple organ dysfunction syndrome and death. Efforts are being made to identify biomarkers for prognosis in SIRS. Sepsis causes an estimated 250,000 deaths annually in the US and 750,000 worldwide. The cost of treating septic patients in an intensive care unit (ICU) can add $5000 or more per day to the cost of a patient's care.

Chromogranin A (CGA) is a biomarker of stress released with catecholamines by the adrenal medulla and has been previously associated with cardiovascular disease and cancer. Serum CGA concentrations are significantly increased in SIRS patients when compared to healthy controls. Highest increase in CGA is seen in patients where infection is associated with SIRS. CGA concentrations positively correlate with biomarkers of inflammation (procalcitonin, CRP) , as well as with Simplified Acute Physiological Score (SAPS). Patients with CGA concentration above 71 μg/L have a significantly shorter survival (Zhang et al. 2009).

Tuberculosis

Mycobacterium tuberculosis is the most common bacterial infection in the world, affecting approximately 2 billion people. This infection is the world's most neglected health problem, killing 3 million people each year – more than all the other infectious diseases combined. Unless diagnosed, active TB is an often-fatal condition, and the patient with active TB will spread the disease to an average of 10–15 others per year. Tuberculosis incidence is increasing in both developed and developing countries. One reason for the sharp increase in TB infections is the development of antibiotic resistant TB strains, including some that are resistant to multiple drugs. World Health Organization (WHO) estimates that this disease will infect 1 billion persons and claim more than 35 million lives in the next 20 years. In the US alone, approximately 15 million residents are infected. Worldwide, one in three persons harbors the causative organism, *M. tuberculosis*. According to WHO, over 1 billion TB tests are performed yearly 2000 and this number is projected to increase.

Tuberculosis is reemerging as an important cause of human disease, particularly in HIV-infected patients who experience severe immunosuppression. Approximately 14% of all cases of TB are associated with HIV, and most tuberculosis infections predominantly involve the lung. Halting the spread of tuberculosis requires a multifaceted approach incorporating early diagnosis, appropriate antimicrobial therapy, proper patient isolation, screening of high-risk populations, and enhanced laboratory biosafety. In the effort to prevent a late-twentieth-century epidemic from becoming a major scourge of the twenty-first century, laboratory personnel and methods will play a key role. Early and prompt diagnosis, particularly in HIV-infected individuals, can reduce the morbidity and mortality of tuberculosis.

Because of the unique nature of this organism, only 10–15% of those infected with *M. tuberculosis* will ultimately develop the disease. Considerable research is planned in this area. In 2010, BioMérieux, Institut Mérieux, and the Singapore government invested $2.2 million to investigate biomarkers to identify individuals who may be at risk for developing tuberculosis and to help guide drug therapies. As part of the project, a joint laboratory is being created at Biopolis in Singapore, where researchers from BioMérieux and Agency for Science, Technology, and Research's Singapore Immunology Network (SIgN) will study the immune cells in the blood of patients infected with TB but whose disease is inactive. The cells will be compared with those of patients who have active TB and those of healthy controls in order to identify potential biomarkers for TB infection and TB re-activation. In addition to aiding in the diagnosis of the disease, the research could help clinicians assess and monitor patient response to TB treatment and manage those who have developed drug-resistance to *M. tuberculosis*.

Conventional Diagnosis of Tuberculosis

Tuberculosis is generally diagnosed by a combination of generalized and specific symptoms along with findings of various laboratory tests. Two widely used tests are the tuberculin skin test and acid-fast microscopic smear. Although both provide rapid results, neither is especially reliable. Skin testing does not distinguish latent infection from active tuberculosis. In addition, distinguishing *M. tuberculosis* from an atypical mycobacterium can prove difficult under the microscope. For reliable detection, a large number of organisms must be present. Sputum smears are positive in only one-half to three-quarters of cases. Culture to distinguish mycobacteria from atypical forms and to determine antibiotic sensitivity takes as long as 3–6 weeks. This distinction is important because atypical forms of mycobacteria do not respond to conventional antibiotics. This time lag in diagnosis, however, delays both the isolation of this contagious disease and the initiation of treatment. The emergence of multidrug-resistant (MDR) tuberculosis has further aggravated attempts to eradicate this infection. MDR organisms are not only resistant to conventional and antimicrobial therapy, but are also associated with a high mortality and rapid occurrence of death.

Molecular Diagnostics for Tuberculosis

Molecular technology is now available to provide detection, identification, and antimicrobial sensitivity testing of mycobacteria. Ideally, a molecular probe would provide these functions directly in a clinical sample, with the sensitivity of a culture, but in a matter of hours rather than weeks. Such rapid results will be essential to provide optimal care for patients infected with *M. tuberculosis* or other mycobacterium species and to limit the spread of tuberculosis.

The FDA approved the Amplified Mycobacterium Tuberculosis Direct (AMTD) test (Gen-Probe, San Diego, California) in 1996. In various studies where the AFB (acid-fast bacilli) smears were cultured, the sensitivity of AMTD was 85.5% and its specificity was 100%. This test, which combines Gen-Probe's transcription-mediated amplification and HPA technologies, yields results in 4–5 h. It can be used on patients who do not have cultivable *M. tuberculosis* but continue to shed these microorganisms. In addition, AMTD can aid in monitoring patients who have been treated with antitubercular drugs.

Biomarkers for Tuberculosis

Large-scale studies have been initiated aiming to identify biomarkers of *M. tuberculosis* infection and disease. Key finding from recent are that no one factor seems able to explain the complex course of *M. tuberculosis* infection.

Multifactorial analyses have identified a variety of genes and proteins, mostly involved in bacterial persistence or host responses, that offer promise as biomarkers for different disease stages. Candidate biomarkers should differentiate people with active tuberculosis from healthy individuals, normalize with therapy, and reproducibly predict clinical outcomes in diverse patient populations (Wallis et al. 2009). Although a large number of promising candidate biomarkers have been examined to date, few patients in these studies have reached clinically meaningful outcomes, and few of the studies have been conducted to international research standards. The challenge now is to validate the suggested biomarkers being described and then reduce them to clinical practice (Doherty et al. 2009). If this can be done, it offers the possibility of greatly improved clinical management of tuberculosis, allowing segregation of patients and contacts into appropriate treatment regimens.

Diagnosis of tuberculous meningitis (TBM) is difficult. Rapid confirmatory diagnosis is essential to initiate required therapy. The presence of 65 kD heat shock protein (hsp) antigen in the CSF of confirmed and suspected cases of TBM would indicate that the selected protein is specific to *M. tuberculosis* and could be considered as a diagnostic biomarker for TBM.

Biomarkers of Pulmonary Tuberculosis in the Breath

Pulmonary tuberculosis may alter volatile organic compounds (VOCs) in breath because Mycobacteria and oxidative stress resulting from Mycobacterial infection both generate distinctive VOCs. A study was condcted to determine if breath VOCs contain biomarkers of active pulmonary tuberculosis (Phillips et al. 2007). Head space VOCs from cultured *Mycobacterium tuberculosis* were captured on sorbent traps and assayed by gas chromatography/mass spectroscopy (GC/MS). Breath VOCs were assayed by GC/MS in patients hospitalized for suspicion of pulmonary tuberculosis and in healthy controls. Sputum cultures were positive for Mycobacteria in 23/42 and negative in19/42 patients. Pattern recognition analysis and fuzzy logic analysis of breath VOCs independently distinguished healthy controls from hospitalized patients with 100% sensitivity and 100% specificity. The study concluded that volatile biomarkers in breath were sensitive and specific for pulmonary tuberculosis: the breath test distinguished between “sick versus well” i.e. between normal controls and patients hospitalized for suspicion of pulmonary tuberculosis, and between infected versus non-infected patients i.e. between those whose sputum cultures were positive or negative for Mycobacteria. However, since these findings were derived from a comparatively small pilot study, confirmation will require additional studies in larger numbers of patients.

Biomarkers of Viral Infections

Whereas most viral infections can be tested by either immunoassays or by DNA, the latter provides the benefit of an earlier, more specifically accurate test. This is because an immunoassay detects only the presence of an antibody to the virus, which cannot be measured until the immune system has actually produced an antibody in the blood. In diseases such as HIV and hepatitis, antibody generation can lag behind infection by as long as 6 months. DNA tests, on the other hand, look for the antigen or the virus itself; it is not necessary to wait for the body to produce antibodies, thus providing earlier detection. Biochemical tests for infections are more prone to human error and require extensive quality control with each new lot. Biochemical-based identification can take up to several days, compared to just several hours for DNA-based tests.

Viral Hepatitis

Approximately 85% of all acute viral hepatitis cases are due to the familiar hepatitis A-E viruses. For the diagnosis of hepatitis A, D, and E, serologic markers are usually adequate. In contrast, molecular diagnosis is important in hepatitis B and C. The cause of hepatitis infection in nearly 15% of the cases continues to baffle physicians.

Hepatitis A virus (HAV) This is the most common cause of viral hepatitis worldwide. This small, single-stranded RNA virus belongs to the enterovirus genus of the Picornavirus family. HAV infection usually produces a brief illness and does not lead to a chronic carrier state or chronic hepatitis. A PCR-based assay can be used to detect HAV antibodies as well as to differentiate between genotypes I and II. In addition, a modified PCR test can detect intact virus particles while ignoring fragments of genetic material from any virus destroyed during the sterilization process. In this method, the blood product is incubated with an HAV-specific monoclonal antibody before the viral RNA is transcribed, amplified, and identified as DNA. The antibody captures any intact virus and the PCR test then identifies the viral nucleic acid, making a false-positive result less likely.

Hepatitis B virus (HBV) There are approximately 300 million chronic carriers of HBV worldwide, representing a global health care challenge. Exposure to contaminated blood is the major source of infection, but other modes of transmission are possible (e.g. inoculation of the ocular surface during corneal transplants). Chronic HBV infection is responsible for much of the world's liver cirrhosis and is implicated

in a high percentage of cases of liver cancer. Situations in which screening for this virus is warranted include the following:

- Clinical suspicion of hepatitis B.
- Blood donors and blood products.
- Monitoring of responses to vaccination.

Because HBV is difficult to culture, its presence is typically demonstrated by electron microscopy. Although the existence of HBV surface antigen (HbsAg) in serum or plasma indicates HBV infection, detection of HbsAg does not provide information on the replicative activity of the virus. Hepanostika HBsAg Ultra assay (bioMerieux), a CE-approved test launched in Europe/Middle East, offers state-of-the-art sensitivity and excellent specificity for the detection of HBV surface antigen in human plasma or serum. The level of HBV DNA in serum or plasma probably reflects the replicative activity of HBV. Various techniques for detection of HBV DNA have been developed, including hybridization assays (which generate quantitative results but lack sensitivity) and PCR (which offers superior sensitivity but predicts only qualitative results, though these findings are important as treatment guides). Methods for quantitative assessment of HBV DNA include Bayer's HBV DNA assay and Abbott's HBV DNA assay. Monitoring the level of HBV may help identify those individuals who are most likely to respond to antiviral therapy, evaluate the efficacy of therapy, and track the infection and viral burden after therapy. Such monitoring provides several benefits:

- It facilitates the tracking of viral load reduction and enables early identification of relapses.
- HBV RNA level at any given time point is predictive of response to therapy.
- The standardization and reproducibility of the assay, as demonstrated on specimens taken at different times and in different places in clinical trials, are helpful in evaluating test results.

Viral hepatic diseases, especially those induced by the HBV, can progress into more serious pathological outcomes and eventually to hepatocellular carcinoma. A growing body of evidence indicates that many trace elements play important roles in a number of carcinogenic processes that proceed through various mechanisms. Markedly elevated Cu:Zn ratios are found in patients having hepatic cirrhosis or hepatocellular carcinoma. These findings imply that the levels of some trace elements, such as selenium, iron, copper, and zinc, and Cu: Zn ratios, might serve as biomarkers for the increased severity of viral hepatic damage.

Hepatitis C virus (HCV) HCV affects roughly 170 million people around the world. Approximately 5 million persons have chronic HCV infection in the US, 30,000 new infections are diagnosed each year, and 8000 infected patients die. In Europe, the number of patients with chronic HCV infection is estimated to be 5–10 million. The number of HCV-antibody-positive individuals is as high as 10–20 million. Acute HCV infection develops into chronic disease in 85% of all cases, setting the stage for development of liver cirrhosis and hepatocellular carcinoma.

The introduction of the approved immunoassay EIA has reduced the incidence of HCV transmission via blood transfusion. It is the first test administered to patients with clinical liver disease. Another test available for HCV is an immunoblot assay (RIBA-2). Both methods are of limited use, however, because a period of several weeks separates infection and seroconversion. In addition, loss of antibody in some persistently infected individuals has been reported. Another monitoring method, which measures the levels of the liver enzyme alanine aminotransferase (ALT), can also give misleading results because fluctuations do not correlate with the levels of HCV. For example, ALT levels may normalize during therapy despite persistent, detectable levels of HCV RNA. If hepatic damage is minimal or has not developed yet, ALT levels may remain normal during active HCV infection. Illnesses other than HCV (e.g. alcoholism) may also produce abnormal ALT values, complicating diagnosis. Direct detection of HCV is valuable in the following situations:

- Diagnosis of neonates born to a seropositive mother.
- Diagnosis of HCV infection in seronegative individuals.
- Diagnosis in organ transplant recipients.
- Assessment of antiviral therapy with interferon-alpha (IFN-α).

Although the recommended treatment for chronic HCV infection involves a 48-week course of PegIFN-α-2b or PegIFN-α-2a combined with ribavirin (RBV), the therapy cures ~40% of those with HCV, and the response is even lower in African-American populations. In addition to limited efficacy, treatment is often poorly tolerated because of side effects that prevent some patients from completing therapy. For these reasons, identification of a biomarker of response to treatment is a high priority. A genetic polymorphism near the IL28B gene, encoding IFN-lambda-3, has been reported to be associated with an approximately twofold change in response to treatment, both among patients of European ancestry and African-Americans (Ge et al. 2009). Almost 80% of those with the favorable response genotype eradicated the virus, while only about 30% with the less favorable response genotype did so. Because the genotype leading to better response is in substantially greater frequency in European than African populations, this genetic polymorphism also explains approximately half of the difference in response rates between African-Americans and patients of European ancestry. On the other hand, among African Americans who did carry the CC genotype, treatment response was 53.3% – higher than the 33.3% treatment response observed among individuals of European descent who had the TT genotype. The favorable C allele also tended to be found less frequently in those with chronic HCV infections, suggesting a role in overall viral clearance. Unexpectedly though, the authors reported that the C alleles actually appeared to be linked to higher rather than lower baseline viral loads. More research is needed to determine whether the newly identified SNP is a biomarker for other important genetic changes or whether the change itself directly influences treatment outcomes.

IP-10 as an important negative prognostic biomarker of HCV infection; given that it mediates chemoattraction of activated lymphocytes, it is counterintuitive that it correlates with therapeutic nonresponsiveness. Plasma levels of the protein IP-10

predict, prior to therapy initiation, the efficacy of treating chronic HCV infection with PEG-interferon and ribavirin, and a prognostic test has been developed a for HCV based on these results (Casrouge et al. 2011).

Biomarkers of SARS

SARS-associated coronavirus (SARS-CoV) has been confirmed as the pathogen for SARS. Several companies are working to develop diagnostics for SARS virus, which are based on detection of the virus.

Cytokines, growth factors and other markers are important indicators of the inflammatory response to infection. Cytokine profiles can also provide a usefull tool for the diagnosis and management of the SARS disease. Acute infections can cause a rapid increase in cytokine levels, an exaggerated response to a high viral load which can be detrimental to the individual. Huge elevations in a variety of cytokine markers may alert clinicians to the severity of the infection and ensure that priority patients are managed immediately to prevent further spread. Markers that have been elevated in SARS are IFN-g, IL-1b, IL-6, IL-12 and MCP-1. Cytokine response has been shown to affect the mortality and morbidity of the patient with communicable diseases. Biochip array technology (Randox Laboratories) offers a blood testing system that can offer rapid cytokine profiling of patient samples. The system uses a panel approach to diagnostic profiling enabling simultaneous measurement of numerous markers in minutes. Ease of use and minimal operator intervention are key benefits of a system that can measure and quantify many clinical markers. Risk of infection to laboratory personnel is limited as sample handling is fully automated and onboard disposal compartments ensure isolation of contaminated waste. The system boasts a test throughput of over 800 cytokine test results per hour, enabling rapid profiling of numerous patients within the time constraints of the WHO regulations.

Biomarkers of HIV

The major cause of AIDS in the world is the retrovirus HIV type 1 (HIV-1). Worldwide, an estimated 18 million persons are infected with HIV, including more than a million individuals in the United States. HIV infection is predominantly a sexually transmitted disorder, although other modes of transmission (e.g., infected blood transfusion and intravenous drug abuse via infected needles) are well recognized.

Direct detection of HIV-1 is difficult because only a small number of cells harbor the virus, a small number of proviral copies exist in each infected cell, and the viral genome has a tendency toward transcriptional dormancy. Nevertheless, a number of

assays have been developed to detect the presence of HIV-1 infection and quantify the level of virus in the blood of infected individuals:

- EIA tests for the detection and quantification of HIV-1 p24 antigen.
- Western blot.
- Latex agglutination.
- Radioimmunoprecipitation.
- Immunofluorescence for the detection of antibodies to HIV-1.
- Viral cultures for the isolation and semiquantification of HIV-1.

HIV-associated neuropsychological impairment is frequent among HIV-1 infected patients but the incidence of HIV dementia has declined since the introduction of HAART therapy (zidovudine, lamivudine and ritonavir boosted indinavir). Improvement of neurocognitive function parallels by normalization of CSF neural markers (NFL, Tau and GFAP) levels and a decline in CSF and serum neopterin and CSF and plasma HIV-1 RNA levels.

APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G; also known as CEM15, or hA3G) is a novel cellular factor of innate immunity that inhibits HIV replication in vitro by causing G to A hypermutations, and consequently reduced relative infectivity of each virus produced by infected cells. Quantification of CEM15 mRNA levels in patient samples as a prognostic indicator of innate HIV/AIDS disease resistance and predicting whether a viral infected patient will be categorized as a long term nonprogressor (LTNP) , which has a much slower disease progression rate. This also provides a method of predicting the level of CD4 cells in a patient, as well as a method of optimizing antiviral therapy in a viral infected patient and has significant implications on new development of diagnostic tools and therapeutic targets to treat viral infections.

Biomarkers in Parasitic Infections

Parasitic infections are still endemic in developing countries. Role of biomarkers in two common parasitic infections, malaria and schistosomiasis, will be discussed here. Biomarker studies in parasitic infections are complicated by the simultaneous infection with multiple parasites in the same individual.

Role of Biomarkers in Malaria

The incidence and severity of malaria infection continue to be on the rise in many parts of the world. The situation is exacerbated by the emergence of multidrug resistance to *Plasmodium falciparum* and *P. vivax*, the two most important human malaria parasites. Malaria is a complex infectious disease in which the host response to infection is dependent upon the parasite stage, parasite virulence factors, and host

genetic background. Diagnosis can be established by identification of the parasite in blood smears. There is still a need to understand the molecular processes that regulate transcriptional activity and gene networks involved in the pathogenesis of or protection from disease as they may provide insights into protective mechanisms of immunity that aid in the design of more effective vaccines.

An analysis of the gene expression profiles has identified a set of host biomarkers, which distinguish between lethal and nonlethal blood stage murine malaria infections with *P. yoelii*. Multiple biological replicates sampled during the course of infection were used to establish statistically valid sets of differentially expressed genes. Genes that correlated with the intensity of infection were used to identify pathways of cellular processes related to metabolic perturbations, erythropoiesis, and B-cell immune responses and other innate and cellular immune responses. Provide insights into transcriptional regulatory mechanisms that influence both the pathogenesis of disease and the host's recovery from infection. While immune responses in human *P. falciparum* and *P. vivax* malaria may share many similar features of the global gene expression program observed in murine malaria, important differences in expression profiles in humans infected clinically or experimentally with malaria will depend on the type of tissue (peripheral blood, bone marrow, spleen, or brain) and the stage of infection (early asymptomatic versus clinical malaria) that is studied.

Because acquisition and maintenance of antimalarial antibodies depend on exposure to malaria infection, such antibodies might be used as biomarkers of transmission intensity. Measurement of these antiboies by serological tests may detect variations in malaria transmission over time and will be invaluable for monitoring trends in malaria endemicity and the effectiveness of malaria control programs (Drakeley et al. 2005). Molecular biomarkers have been investigated for assessing resistance to antimalarial drugs but no conclusive information is available as yet. Efforts to use plasma levels of sTNF-R75 and circulating parasite DNA to estimate sequestered loads of *P. falciparum* have not been successful so far.

It is important to identify individuals infected with P. falciparum who are at risk of developing serious complications such as cerebral malaria. Serum angiopoietin-1 and the angiopoietin-2/1 ratio are promising clinically informative biomarkers for cerebral malaria (Lovegrove et al. 2009). Further studies should address their usefulness as prognostic biomarkers and potential therapeutic targets in severe malaria.

Identification of Biomarkers in Schistosomiasis Infections

Schistosomiasis is the second most prevalent human parasitic disease after malaria and affects more than 200 million people worldwide. The eggs produced when infected by *Schistosoma mansoni* produce complex and unique protein- and lipid-linked glycans, which are important activators and modulators of the host's immune response.

Current diagnosis of schistosomiasis is not ideal. The egg detection by microscopy is specific, but lacks sensitivity and suffers from highly fluctuating egg output. Antibody-based diagnosis is sensitive but fails to reliably identify active infections. Antigen-detection based assays have a number of advantages but fail to detect minimal infections. This has prompted search for biomarkers of the disease.

Scientists have discovered that in addition to the glycoprotein and glycolipid antigens, Schistosoma eggs also excrete unique unconjugated oligosaccharides, which can be identified by using an affinity purification method based on a specific antiglycan MAb. These oligosaccharides appear as biomarkers of infection in the urine of infected individuals and can be detected by mass spectrometry. The identification of new small molecule biomarkers may lead to a new egg-load related assay for light infections in schistosomiasis but may also be used as a measure of infection and morbidity.

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Chapter 10
Biomarkers of Genetic Disorders

Introduction

There are a large number of genetic disorders where molecular diagnostics are used along with biomarkers for screening and diagnosis. These are described in more details in the report on molecular diagnostics (Jain 2017). A few examples will be described briefly here with focus on biomarkers.

Biomarkers of Down's Syndrome

Down's syndrome is a genetic disorder caused by the inheritance of three copies of the 21st chromosome. It is the most common congenital disorder with impairment of mental function; a large percentage of these individuals develop Alzheimer's disease in the fifth decade of life. There is some controversy about the best approach to screening for Down's syndrome. The competing claims of advocates of different screening approaches have made it difficult for health planners, clinicians, or pregnant women to reach a balanced decision about what should be offered, or chosen.

Serum tests used to screen for Down's syndrome include β-human chorionic gonadotrophin (hCG), alpha-fetoprotein (AFP), unconjugated oestriol (uE3), serum pregnancy associated plasma protein-A (PAPP-A), and dimeric inhibin A. ADAM12, a novel serum biomarker properties similar to PAPP-A. In several studies, levels of biomarkers such as complement factor H, Transthyretin6, complement factor B, alpha-1B-glycoprotein44, arylsulfatase A5, and apolipoprotein E45 were shown to be altered in maternal serum from women with DS-affected pregnancies (Yao et al. 2016). These biomarkers might have diagnostic value

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Various studies have advanced our knowledge of the efficacy and safety of antenatal screening for fetal Down's syndrome and placed choices on a firmer platform of evidence. The best performer is an integrated test, comprising ultrasound measurement of fetal nuchal translucency and assay of PAPP-A at 10 weeks, combined with quadruple tests of serum α-fetoprotein, unconjugated oestriol, hCG, and inhibin-A during the second trimester (after 14 weeks). This two step package has a false positive rate of only 0.9%. The best first trimester screening package is a combination of nuchal translucency scan, serum free β-HCG, and pregnancy associated plasma protein A, which has a false positive rate of 4.3%. Second trimester quadruple testing alone has a false positive rate of 6.2%.

Quadruple Marker Prenatal Screening Test (Laboratory Corporation of America) is a blood screening test done in the second trimester of pregnancy (between 15 and 20 weeks) to help detect an increased risk for Down's Syndrome, trisomy 18, and neural tube defects or abdominal wall defects. Occasionally, the test may also detect a risk for other chromosome abnormalities. This test measures the concentrations of four biochemical substances produced by the fetus and placenta, AFP, hCG, uE3, and dimeric inhibin A. The test values, together with maternal age, are then entered into a mathematical formula to determine the risk for the various abnormalities. By adding a fourth marker to the prenatal screening test, the detection rate for an elevated risk of Down's syndrome can be increased from 60% to 75%.

Biomarkers of Muscular Dystrophy

Duchenne and Becker muscular dystrophy (DMD and BMD) share clinical symptoms like muscle weakness and wasting but differ in clinical presentation and severity. Immunohistochemistry using antibodies to dystrophin is the pathological basis for the diagnosis of DMD and BMD. While the sarcolemma of DMD muscle is negative, BMD muscle generally shows variable labeling because of the translation of a partially functional dystrophin that is localized to the sarcolemma. In some cases this differentiation is not possible. In such instances immunolabeling with antibodies to the neuronal form of nitric oxide synthase (nNOS) can be useful in suspecting a dystrophinopathy with a mutation in the 'hot-spot' rod domain and help to direct molecular analysis. nNOS localizes to the sarcolemma of mature muscle fibers via several components of the dystrophin-associated protein complex including dystrophin but sarcolemmal nNOS is lost when dystrophin levels are very low or absent because of deletions in critical regions of the rod domain.

Gene expression profiling of hind limb muscles of mouse models of muscular dystrophies have been shown to clearly discriminate between severely affected and mildly or nonaffected mouse models. Dystrophin-deficient and sarcoglycan-deficient profiles are remarkably similar, sharing inflammatory and structural remodeling processes. These processes are also ongoing in dysferlin-deficient animals, although at lower levels, in agreement with the later age of onset of this muscular dystrophy. The inflammatory proteins Spp1 and S100a9 were up-regulated in

all animal models. Biomarker genes for which expression correlates with the severity of the disease have been identified. Comparative studies are an important step toward the development of an expression profiling-based diagnostic approach for muscular dystrophies in humans.

Biomarkers of Phenylketonuria

Phenylketonuria (PKU) is a genetic disease affecting 1:10,000 to 14,000 live births. In this condition, phenylalanine hydroxylase (PAH) deficiency is inherited as an autosomal recessive trait and the associated hyperphenylalaninaemia phenotype is highly variable. Neurological abnormalities in phenylketonuria include tremor, clumsiness, epilepsy, spastic paraparesis and intellectual impairment. Screening for PKU was introduced in the UK over 30 years ago and has proved successful in preventing severe mental retardation. Genotype-based prediction of the biochemical phenotype is now feasible in the majority of newborns with hyperphenylalaninemia, which may be useful for refining diagnosis and anticipating dietary requirements. Methods currently used to screen for PKU include spectrophotometry, fluorometry, immunoassay, and tandem mass spectrometry with electrospray ionization. Developments in tandem mass spectrometry have made it technically possible to screen for several inborn errors of metabolism in a single analytical step. NeoLynx Screening Application-Manager (Waters Corporation) is indicated for the quantitative measurement of phenylalanine and tyrosine in neonatal blood samples by tandem mass spectrometry – exclusively with Quattro micro/Quattro LC mass spectrometers. Additionally, measurements of tyrosine can be used as an adjunct to the measurement of phenylalanine in reducing the number of false-positive results with NeoLynx Screening Application-Manager.

Genetic Biomarkers of Psoriasis

Psoriasis is a common, immune-mediated genetic disorder of the skin and is associated with arthritis in ~30% of cases. PSORS2 (psoriasis susceptibility locus 2) has been localized to chromosomal region 17q25.3-qter after a genome-wide linkage scan in a family of European ancestry with multiple cases of psoriasis and psoriatic arthritis. In caspase recruitment domain family, member 14 (CARD14), the same authors identified unique gain-of-function mutations that segregated with psoriasis by using genomic capture and DNA sequencing (Jordan et al. 2012). The mutations altered splicing between CARD14 exons 3 and 4. CARD14 activates nuclear factor kappa B (NF-kB), and compared with wild-type CARD14, the p.Gly117Ser and p.Glu138Ala substitutions were shown to lead to enhanced NF-kB activation and upregulation of a subset of psoriasis-associated genes in keratinocytes. These genes included chemokine (C-C motif) ligand 20 (CCL20) and IL-8. CARD14 is localized

mainly in the basal and suprabasal layers of healthy skin epidermis, whereas in lesional psoriatic skin, it is reduced in the basal layer and more diffusely upregulated in the suprabasal layers of the epidermis. The authors propose that, after a triggering event that can include epidermal injury, rare gain-of-function mutations in CARD14 initiate a process that includes inflammatory cell recruitment by keratinocytes. This perpetuates a vicious cycle of epidermal inflammation and regeneration, a cycle which is the hallmark of psoriasis.

Finding the genes responsible for psoriasis will provide a logic framework against which future advances in treatment and/or prevention are likely to evolve. A novel immunoglobulin superfamily gene cluster and a putative RUNX1 binding site variant in the 17q region have also been implicated for psoriasis susceptibility. A USPTO application (#20050277587) titled "CD7 as biomarker and therapeutic target for psoriasis" mentions an extended 5-generation family in which almost half of the family members were affected with psoriasis and/or pityriasis rubra pilaris (PRP) with an autosomal dominant inheritance pattern. A genome-wide scan mapped the disease locus to the terminal part of chromosome 17 and identified the CD7 gene in that region as the gene responsible for psoriasis/PRP in this family. The identification of the gene and its associated pathways/proteins open an avenue of therapeutic targets for drug development in psoriasis with the hope that a more specific and effective therapy can be developed.

Biomarkers of Lysosomal Storage Disorders

Biomarkers of Niemann-Pick Disease

Although several therapies are available or in development for lysosomal storage disorders (LSDs), assessment of therapeutic efficacy is limited by the lack of biomarkers to assess disease progression and severity. This is particularly true for rare diseases such as LSDs, since natural history data from human populations are often lacking. Gene expression analysis in the acid sphingomyelinase-deficient mouse model (ASMKO) of Types A and B Niemann-Pick disease (NPD) has been used to identify novel serum biomarkers. Microarray and real-time PCR analyses are used to compare mRNA expression in ASMKO and normal mice in two important sites of pathology, lung and brain, and several potential biomarkers have been identified and validated from these data. The cytokine MIP-1α is markedly elevated in ASMKO mouse serum, and following enzyme replacement therapy (ERT) it is reduced to normal levels. Total iron levels are similarly raised in ASMKO mice, reflective of the elevated ferritin light chain transcript, and decrease to normal after ERT. Serum growth hormone levels are also elevated in ASMKO mice and were reduced to normal after brain-directed gene therapy, but not ERT. These findings illustrate the value of gene expression analysis for the identification of biomarkers, and provide new insight into the pathobiology of NPD.

Bile Acids as Biomarkers for the Early Diagnosis of NPD

A rapid UPLC-MS/MS method has been used to quantifynovel bile acids in biological fluids and the evaluation of their diagnostic potential in NPC). Two new compounds, NPCBA1 (3β-hydroxy,7β-N-acetylglucosaminyl-5-cholenoic acid) and NPCBA2 (probably 3β,5α,6β-trihydroxycholanoyl-glycine), were found to accumulate preferentially in NPC patients: median plasma concentrations of NPCBA1 and NPCBA2 were 40- and 10-fold higher in patients than in controls (Mazzacuva et al. 2016). Furthermore, NPCBA1 concentrations were normal in some patients because they carried a common mutation inactivating the GlcNAc transferase required for the synthesis of this bile acid. NPCBA2, not containing a GlcNAc moiety, is thus a better NPC biomarker.

Cholesterol Oxidation Products as Biomarkers of NPD

Studies implicating oxidative stress in NPD type 1 (NPD1) pathogenesis have raised the possibility that nonenzymatic formation of cholesterol oxidation products could serve as disease biomarkers. These metabolites were measured in the plasma and tissues of the NPD1−/− mouse model and found several cholesterol oxidation products that were elevated in NPD1−/− mice, were detectable before the onset of symptoms, and were associated with disease progression (Porter et al. 2010). Nonenzymatically formed cholesterol oxidation products were similarly increased in the plasma of all human NPD1 subjects studied and delineated an oxysterol profile specific for NPD1 disease. This oxysterol profile also correlated with the age of disease onset and disease severity. The authors further show that the plasma oxysterol markers decreased in response to an established therapeutic intervention in the NPD1 feline model. These cholesterol oxidation products are robust blood-based bimarkers for NPD1 that may transform diagnosis and treatment of this disorder, and used for monitoring response to therapy.

Biomarkers of Mucopolysaccharidoses

The mucopolysaccharidoses (MPS) is another group of LSDs presenting with broad multi-system disease and a continuous range of phenotypes. MPS' result from primary defects in lysosomal enzymes involved in the degradation of glycosaminoglycans (GAGs). Depending on the specific enzyme defect, the catabolism of one or more GAGs is blocked leading to accumulation in tissues and biological fluids. GAG measurements are important for high-risk screening, diagnosis, monitoring treatment efficacy, and patient follow up. Currently, there are no objective biomarkers of MPS disease that clearly reflect disease severity or therapeutic responsiveness.

Proteomic Technologies for Biomarkers of MPS

Formation of the heparin cofactor II-thrombin (HCII-T) complex, a well-known serine protease inhibitor (serpin)-serine protease complex, has been identified as an informative biomarker for MPS I by using proteomic studies in the murine MPS I model. HCII-T complex was also elevated in plasma from MPS I patients. The degree of HCII-T complex formation appears to correlate with disease severity and is responsive to therapy. In addition to its role as a biomarker, the discovery of increased serpin-serine protease complex formation provides a valuable insight into possible pathophysiological mechanisms of MPS.

Gaucher's disease is the most common LSD. Gene defect leads to deficiency or decreased activity of glucocerebrosidase followed by the accumulation of glucosylceramide. Frequent manifestations are hepatosplenomegaly, anemia, skeletal and hematological abnormalities. Recently used enzyme replacement therapy (imiglucerase) seems to eliminate the need for bone marrow transplantation and has favorable effects on symptoms and outcome. Development of gene therapy (reintroduction of missing DNA sequence) offers the possibility of cure of the disease. The biomarkers secreted by Gaucher's cells are numerous, but none of those identified to date has offered all the expected qualities of a biomarker. Chitotriosidase and chemokine CCL18 are the most useful biomarkers to follow enzyme replacement therapy. Identification of new biomarkers in the future should enable a clearer understanding of the pathophysiology of this complex disease, which involves numerous cell processes.

A reliable tandem mass spectrometry multiplex analysis is used for the quantitation in urine of four GAGs; (1) dermatan sulfate (DS); (2) heparan sulfate (HS); (3) keratan sulfate (KS); and (4) chondroitin sulfate (CS) for eventual technological transfer to the clinic. The developed method is rapid (7 min) and the results show good intraday and interday and accuracy (Auray-Blais et al. 2016). In comparison with the DMB spectrophotometric method, this multiplex tandem mass spectrometry method allows GAG fractionation, thus a differentiation of MPS types, except for MPS I and II which are characterized by the same GAG profile. The devised method is a useful and reliable tool for diagnosis of MPS patients, as well as their monitoring and follow up, as shown by longitudinal studies.

The mucopolysaccharidoses (MPS) result from attenuation or loss of enzyme activities required for lysosomal degradation of the glycosaminoglycans, hyaluronan, heparan sulfate, chondroitin/dermatan sulfate, and keratan sulfate. This review provides a summary of glycan biomarkers that have been used to characterize animal models of MPS, for diagnosis of patients, and for monitoring therapy based on hematopoietic stem cell transplantation and enzyme replacement therapy. Recent advances have focused on the non-reducing terminus of the glycosaminoglycans that accumulate as biomarkers, using a combination of enzymatic digestion with bacterial enzymes followed by quantitative liquid chromatography/mass spectrometry. These new methods provide a simple, rapid diagnostic strategy that can be applied to samples of urine, blood, cerebrospinal fluid, cultured cells and dried

blood spots from newborn infants. Analysis of the non-reducing end glycans provides a method for monitoring enzyme replacement and substrate reduction therapies and serves as a discovery tool for uncovering novel biomarkers and new forms of mucopolysaccharidoses.

Glycan-based Biomarkers for MPS

Glycan biomarkers that have been used to characterize animal models of MPS, for diagnosis of patients, and for monitoring therapy based on hematopoietic stem cell transplantation and enzyme replacement therapy have focused on the non-reducing terminus of the glycosaminoglycans that accumulate as biomarkers, using a combination of enzymatic digestion with bacterial enzymes followed by quantitative LC/MS (Lawrence et al. 2014). These methods provide a simple, rapid diagnostic strategy that can be applied to samples of urine, blood, CSF, cultured cells and dried blood spots from newborn infants. Analysis of the non-reducing end glycans provides a method for monitoring enzyme replacement and substrate reduction therapies and serves as a discovery tool for uncovering novel biomarkers and new forms of mucopolysaccharidoses.

Biomarkers of LSD

Fucosidosis, another LSD, is an autosomal recessive disorder resulting from a deficiency of α-L-fucosidase, encoded by the FUCA1 gene, which leads to failure in the catabolism of glycoproteins and glycosphingolipids resulting in the accumulation of a range of fuco-oligosaccharides and sphingolipids in all tissues including brain and liver. Severely affected patients present within the first year of life with mental retardation, growth retardation, and abnormalities in various other organs. Most of the diagnostic procedures are either invasive or impractical. PCR can be useful only once the mutation is known. The most practical test is detection by mass spectrometry of the elevated oligosaccharides as a biomarker in urine.

Prenatal Diagnosis of LSD

Prenatal diagnosis is available for many LSDs using chorionic villus samples or amniocytes. Such diagnoses can be problematical if sample transport and culture are required prior to analysis. It is possible to identify useful biomarkers for the diagnosis of LSDs from amniotic fluid. Amniotic fluid samples from control and LSD affected pregnancies have been analysed for the protein markers LAMP-1 and saposin C by ELISA, and for oligosaccharide and lipid metabolite biomarkers by electrospray ionization-tandem mass spectrometry. LSD samples included aspartylglucosaminuria, galactosialidosis, Gaucher disease, GM1 gangliosidosis,

mucopolysaccharidosis types I, II, IIIC, IVA, VI, and VII, mucolipidosis type II, multiple sulfatase deficiency, and sialidosis type II. Each disorder produces a unique signature metabolic profile of protein, oligosaccharide, and glycolipid biomarkers. Some metabolite elevations directly related to the disorder whilst others appeared unrelated to the primary defect. Many LSDs are clearly distinguishable from control populations by the second trimester and in one case in the first trimester. Samples from GM1 gangliosidosis and mucopolysaccharidosis type VII displayed a correlation between gestational age and amount of stored metabolite. These preliminary results provide proof of principal for the use of biomarkers contained in amniotic fluid as clinical tests for some of the more frequent LSDs, which cause hydrops fetalis.

Biomarkers of Fabry's Disease

Fabry's disease is a LSD, but it is caused by an X-linked mutation in the gene encoding α-galactosidase. In male patients, the disease has a pronounced vascular phenotype with effects most commonly in the kidney, heart, and brain, owing primarily to storage of globotriaosylceramide (GL-3) within the endothelium; the disease results in renal failure, cardiomyopathy, cardiac events, and strokes. GL-3 clearance in glomerular endothelium has been used as a biomarker endpoint in clinical trials of patients with Fabry disease to determine the efficacy of recombinant enzyme therapy in restoring endothelial cells to near-normal status and prevent renal failure. The FDA accepted the argument that the reduction to normal or near normal in GL-3 accumulation in renal glomerular endothelial cells is reasonably likely to predict clinical benefit, and α-galactosidase A replacement therapy in Fabry's disease received accelerated approval by the FDA after the results of a randomized, placebo-controlled study using biopsy results as an end point.

Renal involvement in Fabry disease is an important criterion of disease prognosis and decision to start early enzyme replacement therapy, which is effective in preventing progression of kidney injury. Gb3 storage, glomerular sclerosis and tubulo-interstitial fibrosis may occur with minimal or no changes on standard renal tests; therefore, alternative biomarkers of renal dysfunction are crucial. A multicenter, prospective, cross-sectional and diagnostic study on patients with Fabry disease and healthy controls has compared several biomarkers with albuminuria for identification of incipient Fabry nephropathy (Aguiar et al. 2017). Biomarkers of glomerular (transferrin and type IV collagen) and tubular (α1-microglobulin, N-acetyl-β-glucosaminidase and alanine aminopeptidase) dysfunction increased in Fabry patients, even in the subgroup without evidence of nephropathy. Inverse significant correlations between estimated glomerular filtration rate (GFR) and collagen type IV or N-acetyl-β-glucosaminidase were stronger than with albumin.

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Chapter 11
Biomarkers of Aging

Introduction

In reference to humans, the process of aging referes to getting old. The World Health Organization terminology for the aging process is as follows:

- 51–60 years: aging persons
- 61– 75 years: elderly persons
- 76–90 years: aged persons
- 91–100 years: very old persons
- 100 years and over: long-lived persons

The age limit of 65 years arbitrarily marks the beginning of old age. This corresponds fairly well with the age of retirement in most countries. There is decline of various body functions and increased predisposition to some diseases, but these manifestations vary considerably from one person to another. Assessment of these changes has led to search for biomarkers of aging. A classification of biomarkers of aging is shown in Table 11.1.

A wide range of biomarkers, reflecting activity in a number of biological systems (e.g. neuroendocrine, immune, cardiovascular, and metabolic), have been found to prospectively predict disability, morbidity, and mortality outcomes in older adult populations. Levels of these biomarkers, singly or in combination, may serve as an early warning system of risk for future adverse health outcomes. In one investigation, several biomarkers were examined as predictors of mortality occurrence over a 12-year period in a sample of men and women 70–79 years of age at enrollment into the study (Gruenewald et al. 2006). Biomarkers examined in analyses included markers of neuroendocrine functioning (epinephrine, norepinephrine, cortisol, and dehydroepiandrosterone), immune activity (C-reactive protein, fibrinogen, IL-6, and albumin), cardiovascular functioning (systolic and diastolic blood pressure), and metabolic activity as indicated by high-density lipoprotein (HDL) cholesterol, total to HDL cholesterol ratio, and glycosylated hemoglobin. Recursive partitioning techniques were used to identify a set of pathways, composed of combinations of

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Table 11.1 Biomarkers of aging

Biomarkers for determining biological age
Epigenetic clocks as determinants of biological age
Gene variants as determinants of biological age
Gene expression profiles for calculating transcriptomic age
Transcriptomic predictors of biological age
Biomarkers of healthy aging
Biomarkers as predictors of mortality with aging
Physiological measurements
Core body temperature
Blood pressure
24-hour energy expenditure
Endocrinological biomarkers
Dehydroepiandrosterone sulfate
Insulin levels
Hypothalamo-pituitary-thyroid axis: thyroid hormone levels
Genes as biomarkers
DNA damage
DNA methylation
Variants located near TERC gene
Mitochondrial mutations
Telomere attrition
Metabolomic biomarkers
Protein biomarkers
Inherent protein aggregation in the absence of neurodegenerative disorders
Advanced glycation end products (AGEs): e.g. carboxymethyl-lysine

different biomarkers that were associated with a high-risk of mortality over the 12-year period. Of the 13 biomarkers examined, almost all entered into one or more high-risk pathways although combinations of neuroendocrine and immune markers appeared frequently in high-risk male pathways, and systolic blood pressure was present in combination with other biomarkers in all high-risk female pathways. These findings illustrate the utility of recursive partitioning techniques in identifying biomarker combinations predictive of mortal outcomes in older adults, as well as the multiplicity of biological pathways to mortality in elderly populations.

Advanced glycation end products (AGEs) have been associated with cardiovascular mortality and impaired renal function in diabetes as well as in uremia in aging individuals (Semba et al. 2009). Synvista Therapeutics is investigating carboxymethyl-lysine as a biomarker of diseases associated with aging. Once this relationship is understood, the company will develop a diagnostic test to follow its level in blood to better determine the utility of medications.

Advances in the molecular biology of aging have yielded a host of candidate as biomarkers of aging. All rely on molecular changes linked to aging, but must still

overcome confounding factors such as individual variation and disease, which may speed or slow aging. If effective anti-aging interventions are to be identified for human application, then the development of reliable and valid biomarkers of aging are essential for this progress. The molecular biomarkers of aging should more accurately predict the physiological age of an organism than the chronological age. The difficulties in establishing useful biomarkers include the biological variation between individuals that makes generalizations difficult; the overlapping of aging and disease processes; uncertainty regarding benign versus pathogenic age-related changes; and the point at which a process begins to damage to the organism. This is being addressed in studies of biomarkers of biological age.

Biomarkers of Biological Age

Biological aging is a better determinant of aging process than chronological age. Several candidate biomarkers of human aging have been proposed, but in most of them vary considerably in cross-sectional studies because of multi-causal and multi-system nature of the aging. Biomarkers of biologic age being examined in the MARK-AGE study, a large, collaborative study across Europe sponsored by the European Commission, which has recruited >3000 persons aged 35–74 (Bürkle et al. 2015). The researchers are collecting data on a number of biomarkers, including DNA-based markers, markers based on proteins and their modifications, immunological markers, clinical chemistry markers, hormones, markers of metabolism, and oxidative stress markers. The massive amount of data has posed data analysis and bioinformatic challenges.

In most such studies, learning something unique about the oldest group of people requires an understanding of what is going on in younger age groups as well. In addition to recruiting across a wide age range, the researchers are selecting people from long-lived families, such as 90-year-old sibling pairs, and some of the participants will also undergo a longitudinal phase of the study. Finally, the researchers have recruited a small number of participants with aging-related genetic disorders, such as progeria (accelerated aging). The aim of the study is to identify a set of biomarkers of aging which, as a combination of parameters with appropriate weighting, would measure biological age better than any single biomarker alone.

A review has considered six potential types of biological age predictors: (1) epigenetic clocks; (2) telomere length; (3) transcriptomic predictors; (4) proteomic predictors; (5) metabolomics-based predictors; and (6) composite biomarker predictors (Jylhävä et al. 2017). Combinations of these various types of predictors may shed light on the aging process and provide further understanding of what contributes to healthy aging. The most promising, biological age predictor is the epigenetic clock; but its value as a biomarker of aging requires longitudinal confirmation.

Gene Variants as Determinants of Biological Age

Definitive gene variants, identified in humans during a whole genome study of mean leukocyte telomere length and located near the TERC gene, have been associated with biological ageing (Codd et al. 2010). An association with telomere length was identified on 3q26 (rs12696304) at a locus that includes TERC, which encodes the telomerase RNA component. This study suggests that some people are genetically programmed to age at a faster rate. The effect was quite considerable in those with the variant, equivalent to between 3 and 4 years of 'biological aging', as measured by telomere length loss. Genetically susceptible people may age even faster when exposed to proven 'bad' environments for telomeres like smoking, obesity or lack of exercise, and end up several years biologically older or succumbing to more age-related diseases. Thus the risk of age-associated diseases including heart disease and some types of cancers are more closely related to biological rather than chronological age.

Gene Expression Profiles for Calculating Transcriptomic Age

Whole-blood gene expression meta-analysis in 14,983 individuals of European ancestry has identified 1497 genes that are differentially expressed with chronological age (Peters et al. 2015). Among these are genes associated with compounds, called glycosaminoglycans, which are involved in wound healing, healthy joints and nerve development. With age, these agents seem to decline. Gene expression profiles were used to calculate the 'transcriptomic age' of an individual, and showed that differences between transcriptomic age and chronological age are associated with biomarkers linked to aging, such as blood pressure, cholesterol levels, fasting glucose, and body mass index. The transcriptomic prediction model adds biological relevance and complements existing epigenetic prediction models.

Biomarkers of Healthy Aging

There are no reference criteria for assessing healthy aging, which limits comparison of research on aging across studies. To address this issue, researchers have developed functional biomarkers of healthy aging in five domains (Lara et al. 2015):

1. Physiological functioning (i.e., cardiovascular function, lung function, glucose metabolism, and musculoskeletal function)
2. Endocrine function (i.e., hypothalamic-pituitary-adrenal [hpa] axis, sex hormones, growth hormones)
3. Physical capability (i.e., strength, balance, dexterity, locomotion)
4. Cognitive function (i.e., memory, processing speed, executive function)

5. Immune function (i.e., inflammatory markers).

Memory, executive function, and speed of processing information are the key subdomains of cognitive function. Potential applications of this biomarker panel are in epidemiological studies of human aging, in health surveys of elderly persons and as surrogate endpoints in intervention studies that aim to promote healthy aging.

Biomarkers of Longevity

Healthy Aging Index

Longevity-associated genes may modulate risk for age-related diseases and survival. The Healthy Aging Index (HAI), a subphenotype of longevity, can be constructed in many studies for genetic analysis. HAI's association with survival has been investigated in the Cardiovascular Health Study and heritability in the Long Life Family Study (Sanders et al. 2014). Rather than generating a large set of potential biomarkers, the researchers wanted to identify a subset of biomarkers that are very good predictors of mortality and are heritable. The data examined for this study were obtained from the Cardiovascular Health Study, an ongoing study of risk for cardiovascular disease in ~6000 participants aged 65 and older. Through a series of analyses, five indicators of mortality were identified: (1) systolic blood pressure; (2) pulmonary vital capacity; (3) serum creatinine (for kidney function); (4) fasting glucose; and (5) cognitive function (based on the modified Mini-Mental Status Examination). These five indicators were each assigned a score of 0, 1, or 2 based on clinical cutoff, with a maximum Healthy Aging Index score of ten for an individual. Comparison of the worst- and best-tertile scores gave a mortality hazard ratio of 2.62. Cardiovascular Health Study participants with unhealthier index scores (7–10) had 2.62-fold greater mortality than participants with healthier scores (0–2). HAI alone predicted death moderately well and slightly worse than age alone. Prediction increased significantly with adjustment for demographics, health behaviors, and clinical comorbidities. In Long Life Family Study, the heritability of HAI was 0.295, 0.387 in probands, and 0.238 in offspring. HAI deserves to be investigated further as a candidate phenotype for uncovering longevity-associated genes in humans.

Effect of Calorie Restriction on Biomarkers of Longevity

Prolonged calorie restriction (CR) increases life span in rodents. A randomized controlled trial, the Comprehensive Assessment of the Long Term Effects of Reducing Intake of Energy (CALERIE), was conducted to determine if prolonged CR affects biomarkers of longevity or biomarkers of oxidative stress, or reduces metabolic rate

beyond that expected from reduced metabolic mass in humans (Heilbron et al. 2006). The findings suggest that two biomarkers of longevity (fasting insulin level and body temperature) are decreased by prolonged CR in humans and support the theory that metabolic rate is reduced beyond the level expected from reduced metabolic body mass. Subjects on CR had less oxidative damage to their DNA, thought to be a marker of aging at the biochemical and cellular level.

Biomarkers as Predictors of Mortality with Aging

A systematic review of 23 cohort studies of blood biomarkers (because of their noninvasive nature) as predictors of mortality in the middle age (between 50 and 75) found 51 potential biomarkers, of which 20 were identified as actual biomarkers, including 25-hydroxyvitamin D, but for only a few of those 20 were enough data available to enable meta-analysis (Barron et al. 2015). Among the final few studies, the researchers identified three biomarkers that were associated with all-cause mortality: (1) C-reactive protein; (2) white cell count; and (3) NT-proBNP (N-terminal pro brain natriuretic peptide), which is also a good predictor of heart function and failure. There was also evidence that cholesterol fractions, erythrocyte sedimentation rate, fibrinogen, granulocytes, homocysteine, intercellular adhesion molecule-1, neutrophils, osteoprotegerin, procollagen type III aminoterminal peptide, serum uric acid, soluble urokinase plasminogen activator receptor, tissue inhibitor of metalloproteinases 1 and tumour necrosis factor receptor II may predict mortality risk. The authors of this review recommended that these 20 biomarkers should be prioritized as potential predictors of mortality in future studies using standardized protocols and reporting methods, as well as a focus on mortality rather than risk of disease or health status.

Genetic Biomarkers of Aging

Since aging is a genetically programmed, it is controlled by genes, but environmental and epigenetic influences predominate in the second half of life. Genes that are expressed in a wide range of tissues and exhibit an age-dependent, easily quantifiable increase in their expression represent a possible molecular biomarker of aging. Genetic mutations affect many phenotypes in flies, worms, rodents, and humans which share several diseases or their equivalents, including cancer, neurodegeneration, and infectious disorders as well as their susceptibility to them. DNA methylation, a force in the regulation of gene expression, is also one of the biomarkers of genetic damage. The mitotic clock of aging is marked, if not guided, by telomeres, essential genetic elements stabilizing natural chromosomic ends.

Genetic Signatures of Longevity

To be a centenarian is rare as only one in six thousand people live to 100 or older. A genome-wide association study, conducted in centenarians and controls to explore the genetic contribution, found that extreme longevity is associated with a select group of genetic biomarkers (Sebastiani et al. 2012). Using these data, the authors built a genetic model that includes 281 SNPs and discriminated between cases and controls of the discovery set with 89% sensitivity and specificity, and with 58% specificity and 60% sensitivity in an independent cohort of 341 controls and 253 genetically matched nonagenarians and centenarians. Consistent with the hypothesis that the genetic contribution is largest with the oldest ages, the sensitivity of the model increased in the independent cohort with older and older ages. For further validation, they applied the model to an additional, unmatched 60 centenarians resulting in 78% sensitivity, and 2863 unmatched controls with 61% specificity. The 281 SNPs include the SNP rs2075650 in TOMM40/APOE that reached irrefutable genome wide significance and replicated in the independent cohort. Removal of this SNP from the model reduced the accuracy by only 1%. Further in silico analysis suggests that 90% of centenarians can be grouped into clusters characterized by different "genetic signatures" of varying predictive values for exceptional longevity. The correlation between three signatures and three different life spans was replicated in the combined replication sets. The different signatures may help to dissect this complex phenotype into sub-phenotypes of exceptional longevity.

Low Serum Thyroid Hormone Level as Biomarker of Longevity

The hypothalamo-pituitary-thyroid axis has been widely implicated in modulating the aging process. Life extension effects associated with low thyroid hormone levels have been reported in multiple animal models. In human populations, an association was observed between low thyroid function and longevity at old age. But the beneficial effects of low thyroid hormone metabolism at middle age remain elusive. The Leiden Longevity Study compared serum thyroid hormone function parameters in a group of middle-aged offspring of long-living nonagenarian siblings and a control group of their partners (Rozing et al. 2010). When compared with their partners, the group of offspring of nonagenarian siblings showed a trend toward higher serum thyrotropin levels in conjunction with lower free thyroxine levels and lower free triiodothyronine levels Compared with their partners, the group of offspring of nonagenarian siblings show a lower thyroidal sensitivity to thyrotropin. These findings suggest that the favorable role of low thyroid hormone metabolism on health and longevity in model organism is applicable to humans as well.

Metabolomic Biomarkers of Aging

Metabolites present in human blood document individual physiological states influenced by genetic, epigenetic, and lifestyle factors. Using LC-MS quantitative metabolomics, analysis of blood of young individuals was compared with the elderly (Chaleckis et al. 2016). Coefficients of variation (CV) were obtained for 126 blood metabolites of all donors. Fifty-five RBC-enriched metabolites, for which metabolomics studies have been scarce, were highlighted. Results showed that 14 blood compounds had remarkable age-related increases or decreases; they include 1,5-anhydroglucitol, dimethyl-guanosine, acetyl-carnosine, carnosine, ophthalmic acid, UDP-acetyl-glucosamine, N-acetyl-arginine, N6-acetyl-lysine, pantothenate, citrulline, leucine, isoleucine, NAD+, and NADP+. Six of them are RBC-enriched, suggesting that RBC metabolomics is highly valuable for human aging research. Age differences are partly explained by a decrease in antioxidant production or increasing inefficiency of urea metabolism among the elderly. Aging affects some age-related compounds concomitantly. Compounds having moderate to high CV values are often modified. Compounds having low CV values, such as ATP and glutathione, may be related to various diseases because their concentrations are strictly controlled, and changes in them would compromise health. Thus, human blood is a rich source of information about individual metabolic differences.

Mitochondrial Mutations as Biomarkers of Aging

Mitochondrial DNA (mtDNA) deletion mutations are observed in skin samples removed from patients having non-melanoma skin cancer. A panel of mtDNA deletions are found in tumor-free skin adjacent to the tumors, but not in the tumors themselves. The tumor samples are more likely to have full-length mtDNA, with point mutations rather than significant deletions. The mtDNA mutations in the tumor-free skin correlate with the aging process.

All newly identified deletion mutations go into "Mitomap (https://www.mitomap.org/)," a database of all known human mitochondrial genome changes. Unraveling the molecular clues as to why aging cells function differently than young cells requires that we have molecular biomarkers that can be tracked. It will be interesting to see if the mtDNA mutations reported as biomarkers of aging in the skin are found in other tissues as well or found only in tissues exposed to ultraviolet light. Some theories of cellular aging center on mitochondria and decreased energetic capacity resulting from mtDNA mutations. This study supports the use of mtDNA mutations as biomarkers of photoaging in the skin. The newly identified biomarkers will provide another tool for studying mitochondrial damage that contributes to aging and cancer, and for screening compounds for these conditions.

Protein Biomarkers of Aging

Carbamylated Proteins as Biomarkers of Aging

Carbamylation is a nonenzymatic posttranslational modification that is caused by the nonenzymatic binding of isocyanate derived from urea dissociation or myeloperoxidase-mediated catabolism of thiocyanate to free amino groups of proteins. This modification is considered an adverse reaction, because it induces alterations of protein and cell properties. It has been shown that carbamylated proteins increase in plasma and tissues during chronic kidney disease and are associated with deleterious clinical outcomes. A study has shown that carbamylation occurs throughout the whole lifespan in some mammals and leads to tissue accumulation of carbamylated proteins with aging (Gorisse et al. 2016). Because of their remarkably long half-life, matrix proteins, like type I collagen and elastin, are preferential targets. The accumulation rate of carbamylation-derived products is inversely correlated with longevity, suggesting the occurrence of still unidentified protective mechanisms. In addition, homocitrulline accumulates more intensely than carboxymethyl-lysine, one of the major advanced glycation end products, suggesting the prominent role of carbamylation over glycoxidation reactions in age-related tissue alterations. Thus, protein carbamylation may be considered a hallmark biomarker of aging in mammalian species that may significantly contribute in the structural and functional tissue damages encountered during aging.

Proteomic Biomarkers of Muscle Aging

Aging of muscles contributes to both loss of functional autonomy and increased morbidity. Muscle atrophy accelerates after 50 years of age, but the mechanisms involved are complex and likely result from the alteration of a variety of interrelated functions. A top-down differential proteomic approach has been used to identify novel biomarkers after the fifth decade of age (Gueugneau et al. 2014). In addition to total muscle extracts, low-ionic strength extracts were investigated to remove high abundance myofibrillar proteins and improve the detection of low abundance proteins. 2DGE with overlapping IPGs were used to improve the separation of muscle proteins. The results indicate important modifications of cytosolic, mitochondrial and lipid energy metabolism, which may relate to dysfunctions in old muscle force generation. A fraction of the differentially expressed proteins were linked to the sarcomere and cytoskeleton (myosin light-chains, troponin T, ankyrin repeat domain-containing protein-2, vinculin, four and a half LIM domain protein-3), which may account for alterations in contractile properties. Other proteins related to calcium signal transduction (calsequestrin-1, sarcalumenin, myozenin-1, annexins) were also identified. Muscle ageing was further characterized by the differential regulation of several proteins implicated in

cytoprotection (catalase, peroxiredoxins), ion homeostasis (carbonic anhydrases, selenium-binding protein 1) and detoxification (aldo-keto reductases, aldehyde dehydrogenases). Several of the differentially expressed proteins were central for proteostasis, including heat shock proteins and proteins involved in proteolysis (valosin-containing protein, proteasome subunit beta type-4, mitochondrial elongation factor-Tu). This study identified 34 new potential biomarkers, which had not been previously recognized as differentially expressed in old muscles, and each may represent a novel starting point to elucidate the mechanisms of muscle chronological aging in humans.

Role of Humanin in Age-Related Diseases

Humanin (HN) is 24-amino acid mitochondria-associated peptide. Since its initial discovery over a decade ago, a role for HN has been reported in many biological processes such as apoptosis, cell survival, substrate metabolism, inflammatory response, and response to stressors such as oxidative stress, ischemia, and starvation. The observation that levels of HN decline with age has led to studies that show beneficial effects of HN and its potent analogs in many age-related diseases including Alzheimer's disease, stroke, diabetes, myocardial ischemia/reperfusion, atherosclerosis, amyotrophic lateral sclerosis, and certain types of cancer. An association between HN levels, growth hormone/insulin-like growth factor-1 (GH/IGF axis), and life span was demonstrated using various mouse models with mutations in the GH/IGF axis. Humanin may be considered a biomarker as well as a therapeutic target in disorders of aging. A review summarizes the role of HN in aging and age-related diseases (Gong et al. 2014).

Role of Bioinformatics in Search for Biomarkers of Aging

System wide functional and structural changes caused by the aging process encourage the implementation of new bioinformatics search strategies for markers of aging. Combinatorial biomarkers should be particularly favored, as they can quantify processes on multiple levels of biological organization and overcome an otherwise limited ability to access heterogeneities in populations. An even more challenging but rational approach is the development of systems biology models to describe molecular pathways and key networks mechanistically as they relate to age. Such reverse engineered models not only indicate critical and diagnostic components (that is, potential biomarkers) but also should be able to predict the progression of aging through computer simulation.

Aging Biomarkers in a Genetically Homogeneous Population

According to UNESCO's Preservation of Parsi Zoroastrian Project, 31% of the Parsi population in India lives beyond the age of 60, compared to 7% nationally (http://www.unescoparzor.com/). A better understanding of the genetic causes of longevity could have a major impact on the Indian Government's healthcare budget and drug companies' marketing efforts. Affymetrix signed an agreement with Avesthagen Ltd. (Bangalore, India), whereby Affymetrix' microarray technology will be used for the AVESTAGENOME Project™, which will explore the genetic basis of longevity and create a genetic, genealogic and medical database of the Parsi-Zoroastrian population. The use of Affymetrix technology will enable researchers to correlate genes with longevity, as well as neurodegenerative conditions, breast cancer, diabetes and other complex diseases that affect the Parsi community. The Parsi community was selected because of its longevity and its relatively genetically homogeneous population. This project takes a systems biology approach that encompasses not only genotyping but also expression profiling and transcriptomics. The genotyping phase of the project, which began in 2007, consisted of 10,000 samples in the first year. By the middle of 2008, the team had performed expression profiling and transcript mapping experiments across a subset of the samples. By 2011, the only information available was the high percentage of elderly, i.e., 31% over the age of 60 in the Parsi population, which is in contrast to the predominantly young population of India. The project is ongoing. All of the genetic information for The AVESTAGENOME Project™ is being collected following informed consent. Data confidentiality is being maintained as in accordance with the Indian Council of Medical Research guidelines.

Telomere Attrition as Aging Biomarker

Telomeres are tandem-repeated hexamers at the ends of mammalian chromosomes. Telomere shortening is associated with cellular senescence and mean telomere length has emerged as a replicative clock within each population of cells and the tissues and organs they build up in vitro and, consequently, as a biomarker for biological aging in vivo. Chronological aging per se does not parallel biological aging, yet accurate and reliable biomarkers are lacking to distinguish between them. The question remains as to whether telomere dynamics is a determinant or merely a predictor of human biological age over and above chronological aging. Although several reports have suggested a link between telomere attrition and aging phenotypes and disorders, both reference values and a complete set of determinants are missing. In the aged telomere attrition is associated with higher mortality due to infections and cardiovascular diseases. Shortening of leukocyte telomere length (LTL) is associated with atherosclerosis, hypercholesterolemia, hypertension and diabetes mellitus. Longer telomeres are associated with slower aging.

Epidemiological evidence for a causal role of telomeres in aging diseases is challenging current knowledge and needs to be further investigated, preferably in longitudinal studies. In future, better assays will enable researchers to quantify LTL more precisely and accurately. High-throughput systems and advanced statistical techniques will allow epidemiologists to link deep phenotyping in large populations with variations in molecules and genes that could influence telomere length (Sanders and Newman 2013).

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Chapter 12
Nutritional Biomarkers

Introduction

Modern epidemiology suggests a potential interactive association between diet, lifestyle, genetics and the risk of many chronic diseases. As such, many epidemiologic studies attempt to consider assessment of dietary intake alongside genetic measures and other variables of interest. However, given the multi-factorial complexities of dietary exposures, all dietary intake assessment methods are associated with measurement errors which affect dietary estimates and may obscure disease risk associations. For this reason, dietary biomarkers measured in biological specimens are being increasingly used as additional or substitute estimates of dietary intake and nutrient status.

Biomarkers of Nutrition for Development Project

The Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) of the NIH, in collaboration with partners representing the breadth of the global food and nutrition enterprise, created Biomarkers of Nutrition for Development (BOND) project to provide advice to anyone with an interest in the role of nutrition in health. BOND program (https://www.nichd.nih.gov/global_nutrition/programs/bond/) will have two separate but complementary tracks:

1. Translational Track will develop processes to inform various user groups about appropriate selection and use of biomarkers; development of translational materials will then generate a research agenda that will support the next track.
2. Research Track will support discovery of biomarkers and development of their use.

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Both tracks will ultimately lead to evidence-based guidance specific to the needs of specific users, communities, and contexts. To accomplish this objective, expert panels are recruited to evaluate the literature and to draft comprehensive reports on the current state of the art with regard to specific nutrient biology and available biomarkers for assessing nutritional status at the individual and population levels. Phase I of the BOND project includes the evaluation of biomarkers for six nutrients: iodine, folate, zinc, iron, vitamin A, and vitamin B-12.

Biomarkers in Nutritional Epidemiology

Genetic variation may influence dietary intake and nutrient metabolism and may affect the utility of a dietary biomarker to properly reflect dietary exposures. Although there are many functional dietary biomarkers that, if utilized appropriately, can be very informative, a better understanding of the interactions between diet and genes as potentially determining factors in the validity, application and interpretation of dietary biomarkers is necessary. Some important biomarkers are being applied in nutrition epidemiology to address some associated questions and limitations (Jenab et al. 2009). There is still a need to identify new dietary biomarkers. Nutritional metabonomics can be used as an analytical method to assess metabolic profiles as measures of dietary exposures and indicators of dietary patterns, dietary changes or effectiveness of dietary interventions. Future studies should be integrate high quality dietary intake information, measurements of dietary biomarkers, metabolic profiles of specific dietary patterns, genetics and novel statistical methods to provide important new insights into gene-diet-lifestyle-disease risk associations.

The National Center for Health Statistics at the CDC has carried out nutrition surveillance of the US population for several years by collecting data about dietary intake, and nutritional biomarker. Physicians, health scientists, and policy makers rely on the National Health And Nutrition Examination Survey (NHANES) reference data to compare the nutritional status of population groups, to assess the impact of various interventions, and to explore associations of nutritional status with health promotion or disease prevention. A review has described challenges faced by researchers measuring biomarkers in NHANES and beyond (Pfeiffer et al. 2017). These challenges include use of multiple related biomarkers instead of a single biomarker for a specific nutrient (e.g., folate, vitamin B-12, iron), special needs for specimen collection and handling to ensure optimum specimen quality (e.g., vitamin C, folate, homocysteine, iodine), the retrospective use of long-term quality-control data to correct for assay shifts (e.g., vitamin D, vitamin B-12), and the proper planning for and interpretation of crossover studies to adjust for systematic method changes (e.g., folate, vitamin D, ferritin).

Biomarkers of Nutritional Status

Humans have individual differences in response to diet and nutrition should take into account differences in their genetics and metabolic needs. The available diagnostic biomarkers for disease may not be appropriate or adequate to distinguish the nutritional status of humans or form a basis for recommending appropriate diets for optimal metabolic health. Metabolic profiling by use of metabolomics is required for this purpose. There is a need for measuring metabolites to assess human metabolism prior to onset of disease. The current use of single biomarkers as indicators of disease will be replaced by comprehensive profiling of individual metabolites linked to an understanding of health and human metabolism as determined by metabolomics. Industrial and academic initiatives are currently developing the analytical and bioinformatic technologies needed to assemble the quantitative reference databases of metabolites as the metabolic analog of the human genome. With these in place, dietetics professionals will be able to assess both the current health status of individuals and predict their health trajectories. This will facilitate integration of metabolism with the genetic and dietary variables that affect health and lead to personalized nutritional counseling. There is, however, a paucity of good studies on nutritional biomarkers. Nutritional biomarkers are shown in Table 12.1.

Ferritin as Biomarker of Nutritional Status

Ferritin is an intracellular protein that stores iron and releases it into the serum where it functions as an iron carrier. Plasma ferritin is a biomarker of the total amount of iron stored in the body, and is used as a diagnostic test for iron-deficiency

Table 12.1 Nutritional biomarkers

Biomarker	Significance
Ferritin	Ferritin is an intracellular protein that contains iron. Blood ferritin test can detect iron storage disorders and iron deficiency.
Folate	Folate is required for remethylation of homocysteine to methionine and DNA synthesis.
Iodine	Urinary iodine (UI) is an excellent indicator of recent iodine intake. Iodine is linked to thyroid metabolism and is well correlated with the severity of iodine deficiency.
Vitamin A	Serum retinol, retinol-binding protein and retinyl esters indicate liver vitamin A concentrations, the gold standard for vitamin A status.
Vitamin B12 (cobalamin)	Deficiency causes reversible megaloblastic anemia and demyelinating disease.
Vitamin D	Vitamin D deficiency is linked with increased risk of many chronic diseases.
Zinc	Zinc is involved in several metabolic processes. Interindividual variation in zinc metabolism make it difficult to assess zinc status due to dietary variations and drug effects.

anemia. Low ferritin level is associated with anemia and excess of ferritin can lead to iron overload disorders such as hemochromatosis.

Folate Biomarkers Related to Nutritional Health Status

Folate is required for remethylation of homocysteine to methionine and DNA synthesis and cell proliferation in addition to methylation reactions that affect critical processes such as methylation of cytosine in DNA for control of gene expression and neurotransmitter synthesis. The types of chronic diseases linked to folate status and folate-related metabolic abnormalities include cancer, cardiovascular diseases, stroke, and megaloblastic anemia. Available folate biomarkers include serum folate, RBC folate, and plasma homocysteine concentrations. Some of the conclusions of the study of these biomarkers by the BOND project are (Bailey et al. 2015):

Serum folate Serum folate provides information on the short-term folate status of an individual. It is the earliest indicator of altered folate exposure reflecting recent dietary intake, and is highly responsive to intervention. Natural food folates typically result in a poorer serum folate response than does folic acid supplementation.

RBC folate RBC folate is a sensitive indicator of long-term folate status and is highly correlated with folate intake. Natural food folates typically result in a poorer RBC folate response than does folic acid supplementation.

Plasma homocysteine (Hcy) Plasma homocysteine (Hcy) is elevated in folate deficiency. Remethylation of Hcy to methionine is catalyzed by the vitamin B12-dependent enzyme methionine synthase, and several other nutritional cofactors. Thus, plasma Hcy is not a specific biomarker of folate status may be elevated due to lifestyle factors, renal insufficiency, and drugs.

Folate deficiency-specific alterations in gene expression, folate deficiency-specific miRNAs such as miR-222, and proteomic changes in response to folic acid supplementation, may be useful for supporting interpretation of established biomarkers.

Iodine as Biomarker of Nutritional Status

Iodine, like most nutrients, plays an important role in human health. Interactions can occur that affect absorption, metabolism, and function. Effects of iodine deficiency on growth and development are collectively known as iodine deficiency disorders, which result from a deficiency of thyroid hormone. Goitrogens and micronutrient deficiencies that affect iodine metabolism and thyroid function include the following:

- Diet: cruciferous vegetables, soy and millet, linseed, etc.
- Industrial pollutants
- Deficiency of other nutrients: selenium, iron and vitamin A

A BOND project study covers all relevant aspects of iodine biology and currently available biomarkers, which include the following (Rohner et al. 2014):

Urinary iodine (UI) Because >90% of dietary iodine is excreted in the urine, UI is an excellent indicator of recent iodine intake and can be expressed as a 24-h excretion (μg/d), as a concentration (UIC; μg/L), or in relation to creatinine excretion (μg iodine/g creatinine).

Thyroid stimulating hormone (TSH) This is used mainly for newborn screening to detect congenital hypothyroidism; for other population groups, mean TSH values do not reliably discriminate between iodine-deficient and iodine-sufficient populations.

Thyroglobulin Thyroglobulin is the scaffold protein within which T3 and T4 are synthesized, and small amounts may be secreted into the blood along with T3 and T4. Serum thyroglobulin is well correlated with the severity of iodine deficiency as measured by UI. Measurement of thyroglobulin by dried blood spot technology can categorize iodine status of populations

Goiter Ultrasonography for measuring volume of the thyroid gland is objective supplement to clinical examination by inspection and palpation.

Zinc as a Biomarker of Nutritional Status

Zinc is needed for several metabolic processes as a regulatory, or catalytic ion. Zinc homeostasis is regulated for sustaining metabolic functions over a wide range of zinc intakes; therefore, it is difficult to assess lack or excess of zinc. The BOND Zinc Expert Panel recommends three measurements for estimating zinc status: dietary zinc intake, plasma zinc concentration (PZC), and height-for-age in infants and children (King et al. 2016). PZCs respond to severe dietary zinc restriction and to zinc supplementation; they also change with shifts in whole-body zinc balance and clinical signs of zinc deficiency. PZC cutoffs are available for identifying individuals and populations at risk of zinc deficiency. However, there are limitations in using the PZC to assess zinc status as it respond less to additional zinc provided in food than to a supplement administered between meals, there is considerable inter-individual variation in PZCs with changes in dietary zinc, and PZCs are influenced by the time of day, inflammation, and certain drugs.

Biomarkers of Branched Chain Amino Acid Status

Branched chain amino acids (BCAAs) are not synthesized in the body in humans, but they are crucial in protein and neurotransmitter synthesis. The protein anabolic role of BCAAs seems to be mediated not only by their important role as a promoter of the translation process (and possibly acting at the transcription level) but also by

inhibition of protein degradation. Leucine may play a critical role in these signaling pathways. Supplementation with BCAAs spares lean body mass during weight loss, promotes wound healing, may decrease muscle wasting with aging, and may have beneficial effects in renal and liver disease. BCAA supplementation is extensively used in the athletic field with the assumption of improved performance and muscle mass. Measuring serum BCAAs has limited clinical utility beyond the controlled setting because levels are affected by a variety of clinical states, and optimal levels in these scenarios have not been completely elucidated. Diet, hormones, stress, aging, and renal or liver dysfunction affect BCAA levels and our understanding the biological effects of BCAAs may help to develop biomarkers of BCAA status.

Biomarkers of Caloric Restriction

Caloric restriction is associated with a decreased level of oxidative stress. Reactive oxygen species (ROS), generated predominantly in mitochondria, are attenuated by decreased caloric intake (Skrha 2009). On the other hand, antioxidative mechanisms are frequently accelerated by increased gene expression or activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, paraoxonase, etc.). Measurement of biomarkers of oxidative stress in caloric restriction is therefore important in experimental as well as clinical studies. Estimation of ROS in tissues and fluids is typically performed by measurement of oxidant products (i.e. malondialdehyde, F-2-isoprostanes, nitrotyrosine) and biomarkers of antioxidant system (enzymes, glutathione, alpha-tocopherol, ascorbic acid, ubichinone, etc).

Biomarkers of Malnutrition

Malnutrition is a general term for a medical condition caused by an improper or inadequate diet and nutrition. Manifestations may be subclinical. Malnutrition is common in infants in the third world and is a major cause of infant mortality. Nucleotide intake and nutritional recovery has a notable effect on IGF-I, IGF binding protein-3 (IGFBP-3) and other hormonal biomarkers. This outcome could stimulate the catch-up growth of severely malnourished infants and toddlers during the nutritional recovery period.

Malnutrition is found even among the elderly in developed countries. Levels of serum lipids are influenced by malnutrition and inflammation. Total cholesterol, HDL, and LDL can be considered novel biomarkers of malnutrition and inflammation in geriatric patients (Hrnciarikova et al. 2009). A close relation has also been demonstrated between serum lipids and prealbumin. In healthy elderly subjects, plasma transthyretin and alpha 1-acid glycoprotein could be helpful in identifying elderly subjects at higher risk of death (Carriere et al. 2008).

Maternal Nutrition During Early Pregnancy Causes Epigenetic Changes

In experimental animals, maternal diet during the periconceptional period influences the establishment of DNA methylation at metastable epialleles in the offspring, with permanent phenotypic consequences. Pronounced naturally occurring seasonal differences in the diet of rural Gambian women enabled testing of this in humans (Dominguez-Salas et al. 2014). It showed that significant seasonal variations in methyl-donor nutrient intake of mothers around the time of conception influence 13 relevant plasma biomarkers. The level of several of these maternal biomarkers predicts increased/decreased methylation at metastable epialleles in DNA extracted from lymphocytes and hair follicles in infants postnatally. These results demonstrate that maternal nutritional status during early pregnancy causes persistent and systemic epigenetic changes at human metastable epialleles.

Proteomic Biomarkers and Nutrition

Scientists at the Nestlé Research Centre (Lausanne, Switzerland) are employing proteomics to address questions of nutrition and health. Nestlé believes that foods and drinks affect individual consumers differently. A food may be well-tolerated by one individual cause but cause violent gastric discomfort in another. Food preference may be related to biomarkers. It is worthwhile to investigate genes that are activated by specific foods for enhancing health and wellness. Certain individuals are more predisposed than others to conditions like obesity or diabetes. If protein markers that indicate such predisposition can be identified before disease symptoms arise, dietary approaches could be devised for health promotion and disease prevention. Nestlé is now including genomics and proteomics approaches into consumer research to impart the health and wellness dimension and to more accurately address individual differences in terms of response to diet and food preference. The long-term deliverable of "Omics" driven food research is personalized nutrition. Proteomics adapted and applied to the context of nutrition and health has the potential to deliver biomarkers for health and comfort, reveal early indicators of disease disposition, assist in differentiating dietary responders from non-responders, and, last but not least, discover bioactive, beneficial food components.

Vitamin Deficiency as Biomarker of Disease

Deficiency of several vitamins as causes of human diseases is well known. Low serum levels of these vitamins may be considered as biomarkers. Low serum concentrations of vitamins B6 and B12 and selenium are biomarkers that predict

subsequent disability in activities of daily life in older women living in the community. This is one of the key factors to be considered in the development of strategies aimed at preventing or delaying the process of disability. Nutritional biomarkers are shown in Table.

Vitamin A Biomarkers

A thorough review of vitamin A biomarkers has been conducted by the BOND initiative (Tanumihardjo et al. 2016). Some biomarkers of vitamin A status in population studies are as follows:

- Serum/plasma retinol is the most commonly used biomarker. It correlates with the prevalence and severity of xerophthalmia and changes in response to interventions.
- Serum retinol-binding protein is not released from the liver when retinol is limiting. It is used as a proxy for serum retinol in identification of vitamin A deficiency.
- Relative dose response is based on hepatic accumulation of RBP during vitamin A depletion. It requires blood sample before and after an oral retinyl ester dose.
- Retinol isotope dilution, although technically challenging, is the most sensitive test to measure vitamin A status and impact of interventions on vitamin A reserves. It is minimally invasive and accurate.
- Breast-milk retinol is a good indicator of vitamin A status in areas where breastfeeding is common until ≥6 m of age.
- Retinyl esters are validated qualitative measure of hypervitaminosis A.
- Electroretinography measures the bioelectrical response of the retina to a flash of light, but it is invasive and not suitable for children.
- Pupillary threshold testing inversely correlates with serum vitamin A levels in the low-deficient range and the concentration of vitamin A in the retina.

Vitamin B12 Deficiency

Vitamin B12 (cobalamin) deficiency causes reversible megaloblastic anemia, demyelinating disease, or both and is an under recognized problem in the elderly in daily clinical practice. Autoimmune gastritis (pernicious anemia) is the most common cause of severe deficiency. Early detection is important because prolonged severe deficiency of this vitamin can lead to irreversible neurological damage, anemia, osteoporosis, and cerebrovascular as well as cardiovascular diseases. The prevalence of B12 deficiency increases with age and some clinical abnormalities that were previously thought to be related to normal aging changes

may actually be caused by cobalamin deficiency. Detection of B12 deficiency is important because parenteral or high-dose oral vitamin B12 is an effective therapy. Current assays have insufficient sensitivity and specificity for diagnosis of B12 deficiency. However, there are some biomarkers for detection of cobalamin deficiency: RBC mean corpuscular volume; serum cobalamin level; plasma holotranscobalamin; serum methylmalonic acid (MMA) levels and serum total homocysteine (tHcy) levels. A roundtable discussion by NIH reviewed three biomarkers of vitamin B12 status used in past NHANES (National Health and Nutrition Examination Survey) – serum vitamin B12, MMA, and tHcy – and discussed the potential utility of measuring holotranscobalamin (holoTC) for future NHANES (Yetley et al. 2011). The roundtable focused on public health considerations and the quality of the measurement procedures and reference methods and materials that past NHANES used or that are available for future NHANES. Roundtable members supported reinstating vitamin B12 status measures in NHANES. They noted evolving concerns and uncertainties regarding whether subclinical (mild, asymptomatic) vitamin B12 deficiency is a public health concern. They identified the need for evidence from clinical trials to address causal relations between subclinical vitamin B12 deficiency and adverse health outcomes as well as appropriate cutoffs for interpreting vitamin B12-related biomarkers. They agreed that problems with sensitivity and specificity of individual biomarkers underscore the need for including at least one biomarker of circulating vitamin B12 (serum vitamin B12 or holoTC) and one functional biomarker (MMA or tHcy) in NHANES. The inclusion of both serum vitamin B12 and plasma MMA, which have been associated with cognitive dysfunction and anemia in NHANES and in other population-based studies, was preferable to provide continuity with past NHANES. Reliable measurement procedures are available, and National Institute of Standards and Technology reference materials are available for serum vitamin B12 and MMA. Although measurement of MMA, homocysteine, or both is used to confirm vitamin B12 deficiency in untreated patients, an elevated level of MMA is more sensitive and specific for the diagnosis (Stabler 2013).

Vitamin B12 status may affect the brain through multiple mechanisms. In study on elderly community-dwelling persons, serum biomarkers of vitamin B12 status were related to neuropsychological tests and brain MRI measures obtained on average 4.6 years later (Tangney et al. 2011). Concentrations of all vitamin B12-related biomarkers, but not serum vitamin B12 itself, were associated with global cognitive function and with total brain volume. MMA levels were associated with poorer episodic memory and perceptual speed, and cystathionine and 2-methylcitrate with poorer episodic and semantic memory. Homocysteine concentrations were associated with decreased total brain volume. It was concluded that MMA, a specific biomarker of B12 deficiency, may affect cognition by reducing total brain volume whereas the effect of homocysteine (nonspecific to vitamin B12 deficiency) on cognitive performance may be mediated through increased white matter hyperintensity and cerebral infarcts.

Vitamin D Deficiency as a Biomarker of Disease

Vitamin D status is linked with increased risk of many chronic diseases including autoimmune diseases, cardiovascular disease, cancers, type II diabetes and infectious diseases. This new awareness of the importance of vitamin D to overall health and the consequences of vitamin D deficiency has stimulated rapid growth in the demand for vitamin D tests at hospitals and clinics. Consequently, antibodies for use in such diagnostic tests are highly sought after within the healthcare industry. The complication for vitamin D tests is that there are two different forms of vitamin D, named D3 and D2, both of which are active. Vitamin D3 is derived within the body from exposure of the skin to sunlight whereas D2 is a yeast- and plant-derived product, available in supplements and fortified foods. There is broad agreement within the medical community that vitamin D tests must recognise the two relevant forms of vitamin D equally. Using its proprietary technology, Bioventix's scientists has created an antibody called vitD3.5H10 that recognizes both relevant forms of vitamin D with equal and high affinity. Commercial scale manufacture has already started and evaluation studies using prototype tests based on vitD3.5H10 are being conducted. It could have significant utility in the manufacture of tests designed to determine the vitamin D status of patients.

Role of Biomarkers in the Development of Personalized Nutrition

Role of biomarkers in the development of personalized medicine is discussed in Chap. 18. Personalized nutrition has been traditionally based on the adjustment of food and diet according to individual needs and preferences. With advances in molecular biology, there is a shift in focus from epidemiology and biochemistry to an understanding of how nutrients act at molecular level. Advances in genomics have led to recognition of the importance of genes in human nutrition. Nutrigenomics or nutritional genomics implies the study of effects of nutrition at the genome level. Nutrigenetics examines the effect of genetic variation on the interaction between nutrition and disease.

The optimal development of evidence-based nutritional guidance to promote health requires an adequate assessment of nutrient bioavailability, bioactivity, and bioefficacy. To achieve this, reliable information about exposure to nutrients, their intake, and functional effects is required; thus, the identification of valid biomarkers using standardized analytical procedures is necessary (Rubio-Aliaga et al. 2012). a comprehensive set to assess the nutritional status and metabolic conditions of nutritional relevance is not yet available. Also, there is very limited knowledge on how the extensive human genetic variability influences the interpretation of these biomarkers. Nutrigenomics, along with other 'omics' such as transcriptomics, proteomics, metabolomics, and epigenomics, is a promising approach for identifying new biomarkers of nutrition.

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Chapter 13
Biomarkers of Cancer

Introduction

Any measurable specific molecular alteration of a cancer cell either on DNA, RNA, protein, or metabolite level can be referred to as a cancer biomarker. The expression of a distinct gene can enable its identification in a tissue with none of the surrounding cells expressing the specific biomarker. In the past decade, molecular dissection of the cancer by means of mRNA expression profiling enabled detailed classification according to tumor subtypes. The traditional system of tumor node metastases (TNM) has been the main tool for identifying prognostic differences among patients and for guiding the treatment. The TNM system is based on the macroscopic and microscopic morphological examination of pathological samples. Despite the advantage of uniformity for international communications and studies, there are many limitations of this system as a first line method for prediction and prognosis of cancer. It is difficult to distinguish related disease subtypes, which have different clinical outcomes. Hence there is a need for more exact molecular biomarkers for use in clinical practice. In recent years the discovery of cancer biomarkers has become a major focus of cancer research. The widespread use of prostate-specific antigen (PSA) in prostate cancer screening has motivated researchers to identify suitable biomarkers for screening different types of cancer. Biomarkers are also useful for diagnosis, monitoring cancer progression, predicting recurrence and assessing efficacy of treatment (Jain 2014). The advent of targeted therapies such as imatinib (Novartis' Gleevec), trastuzumab (Roche's Herceptin) and rituximab (Roche's Mabthera), where a causal relationship has been established between drug target and therapy, drives the need for biomarkers for selecting the patients for a given therapy as well as for predicting drug resistance.

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Table 13.1 Desirable characteristics of biomarkers for cancer

	Characteristics				
Purpose	Noninvasive	Low Cost	Simple to Perform	Accurate[a]	Informative (Discriminatory)
Screening	+++	+++	+++	+++	+++
Predisposition	+++	+++	+++	+++	+++
Early detection	++	++	++	+++	+++
Prognosis	+	+	+	++	++
Drug response	+++	++	++	+++	+++
Target for drug	NA	+	NA	+++	NA

+ = low importance, ++ = medium importance, +++ = high importance, NA = not applicable.
[a]Low rate of false-negative results

The Ideal Biomarker for Cancer

The ideal biomarker for cancer would have applications in determining predisposition, early detection, assessment of prognosis, and drug response. It would be an additional advantage if the biomarker could also serve as a target for drug development. Desirable characteristics of molecular biomarkers for cancer are shown in Table 13.1.

No one test meets all these requirements but these should be kept in mind for selection of diagnostic tests. There is an urgent need for cancer biomarkers with more accurate diagnostic capability, particularly for early stage cancer.

Biomarkers and Hallmarks of Cancer

The hallmarks of cancer comprise 8 biological capabilities acquired during the multistep development of human tumors and constitute an organizing principle for rationalizing the complexities of neoplastic disease (Hanahan and Weinberg 2011):

1. Sustaining proliferative signaling
2. Evading growth suppressors
3. Resisting cell death
4. Enabling replicative immortality
5. Inducing angiogenesis
6. Activating invasion and metastasis
7. Reprogramming energy metabolism (emerging)
8. Escaping immune destruction (emerging)

Underlying these hallmarks are genome instability, which generates the genetic diversity that expedites their acquisition, and inflammation, which fosters multiple hallmark functions. In addition to cancer cells, tumors exhibit another dimension of

complexity: they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the "tumor microenvironment." These hallmarks should be taken into consideration for detection of biomarkers as guide to diagnosis and treatment of cancer.

Single Vs Multiple Biomarkers of Cancer

Because cancer is a polygenic disease, most diagnostic assessments would probably rely on multiple biomarkers. Although few biomarkers may be needed for testing for predisposition, possibly more would be required for early detection, prognosis prediction, and determination of drug response. Such biomarkers might be qualitative for predisposition tests and early detection of lesions, but quantitative for determining differential gene expression (as may be required for determining prognosis and drug response). If gene expression is not confined to a single library, it should be at least eight times the level of expression in the other library to be statistically significant.

Cancer is a product of the tissue microenvironment. Therefore, interactions between the cancer cells, the surrounding epithelial and stromal cells, vascular channels, the extracellular matrix, and the immune system are the ultimate determinant of the final pathology. Cell-surface antigens and receptors, cell-anchored and secreted enzymes, cytokines, and extracellular matrix molecules are the mode of communication between disease cells and the surrounding microenvironment. The outcome is a complex cascade of molecules available for sampling by the ongoing vascular perfusion. Low molecular weight peptides in the blood may comprise a recording of events in the disease microenvironment. The peptidome signature shed from the microenvironment is a reflection of the microenvironment as a whole. In case of cancer biomarkers, cancer specificity is not derived from proteins secreted exclusively by tumor cells. MS profiling indicates that a higher level of both specificity and sensitivity might be achieved by measuring the combination of biomarkers emanating from both the diseased cells and the reactive cells in the microenvironment. In this way the peptidome can potentially supplant individual single biomarkers and transcend the issues of tumor and population heterogeneity. Therefore, trend in biomarker discovery is moving away from the ideal single, cancer-specific biomarker such as prostate specific antigen. Despite decades of effort, most single biomarkers have not reached the level of cancer specificity and sensitivity required for routine clinical use in early detection and screening purposes. A growing confluence of scientific data and results point to combinations of blood-borne markers using MS profiling techniques as well as tissue MS profiling strategies, and multiplexed immunoassay providing more superior results than single markers alone.

Genetic alterations in tumor cells often lead to the emergence of growth-stimulatory autocrine and paracrine signals, involving overexpression of secreted peptide growth factors, cytokines, and hormones. Increased levels of these soluble

proteins may be exploited as markers for cancer diagnosis and management or as points of therapeutic intervention. The combination of annotation/protein sequence analysis, transcript profiling, immunohistochemistry, and immunoassay is a powerful approach for delineating candidate biomarkers with potential clinical significance.

Types of Cancer Biomarkers

Various types of cancer biomarkers are shown in Table 13.2. Biomarkers specific for various cancers are described later in this chapter.

Table 13.2 Types of cancer biomarkers

Genetic biomarkers
Biomarkers of PTEN tumor suppressor gene status
Gene mutations
Oncogenes
Tumor microvesicles or exosomes
DNA biomarkers
DNA repair biomarkers
Gene amplification
Microsatellite instability
Mitochondrial DNA
Viral DNA
RNA biomarkers: microRNAs (miRNAs)
Protein biomarkers
B7 coregulatory ligands
Carcinoembryonic antigen (CEA, CD66e, CEACAM-5)
High motility group protein A2
Raised serum lactate dehydrogenase
YKL-40, a secreted glycoprotein
Metabolic biomarkers
Hypoxia-inducible factor-1
Epigenic biomarkers: DNA methylation
Immunological biomarkers: T cell and cytokine responses
Biomarkers in cancer stem cells: Cripto-1
Circulating cancer biomarkers
Circulating tumor cells as cancer biomarkers
Circulating nucleic acids as biomarkers of cancer: DNA and miRNA
Circulating exosomes or microvesicles

miRNAs as Biomarkers in Cancer

Role of miRNAs in oncogenesis has been investigated. miRNAs can behave like oncogenes, which promote tumor growth, or tumor suppressors, which keep potentially malignant cells in check. miRNA activity is tissue-sensitive, meaning some miRNAs may be overexpressed, or "turned on" in some of the cancers while in others they are underexpressed, or "turned off." Increased expression of miR-21, an oncogene leads to increased human telomerase reverse transcriptase (hTERT) expression and increased telomerase activity causes cell immortalization – characteristic of cancer. Decreased expression of let-7, a tumor suppressor increases expression of oncogene RAS and induces cell proliferation – another feature of cancer.

miRNA genes are frequently located in cancer-associated regions of the genome, e.g. at fragile sites, as well as in minimal regions of loss of heterozygosity, minimal regions of amplification (minimal amplicons), or common breakpoint regions. They can act both as tumor suppressor genes and oncogenes. The classic paradigm in oncogenesis is the accumulation of mutations in the open reading frames of protein-encoding oncogenes and tumor suppressors. The identification of miRNAs that are important for development and cell homeostasis will likely change this paradigm of cancer. The determination of miRNA profiles as a new class of biomarkers has the potential to significantly improve diagnostic accuracy and prognostic information. The full complement of miRNAs in a genome may be extensively involved in cancer However, a small percentage of miRNA genes located in deleted regions had low levels of expression in cancer samples.

Several studies of miRNAs highlight a requirement for cell viability. Posttranscriptional silencing of target genes by miRNAs occurs either by targeting specific cleavage of homologous mRNAs, or by targeting specific inhibition of protein synthesis. A multisubunit protein complex termed 'microprocessor' is necessary and sufficient for processing miRNA precursor RNAs. Microprocessor contains Drosha, an RNase III endonuclease, and DGCR8, a gene deleted in DiGeorge syndrome. These findings link miRNA perturbation to cancer.

One cluster of miRNAs, the mir-17–92 polycistron, is located in a region of DNA that is amplified in human B cell lymphomas and the levels of the primary or mature miRNAs derived from the mir-17–92 locus are often substantially increased in these cancers. Enforced expression of the mir-17–92 cluster has been shown to act with c-myc expression to accelerate tumor development in a mouse B cell lymphoma model. A set of five miRNAs, including the three most up-regulated ones (miR-221, -222, and -146), can distinguish unequivocally between papillary thyroid carcinoma and normal thyroid indicating their involvement in the pathogenesis of carcinoma. Marked overexpression of the miR-17-92 cluster with occasional gene amplification may play a role in the development of lung cancers, especially in their most aggressive form, small-cell lung cancer. Most of the studies indicate that miRNAs, can modulate tumor formation, and implicate mir-17–92 cluster as a potential human oncogene.

A cancer miRNA signature, composed of a large portion of overexpressed miRNAs, has been identified from a large-scale miRnome analysis of samples of several cancers. These miRNAs include some with well characterized cancer association, such as miR-17-5p, miR-20a, miR-21, miR-92, miR-106a, and miR-155. A number of the predicted targets, including the tumor suppressors RB1 (Retinoblastoma 1) and TGFBR2 (transforming growth factor, beta receptor II) genes have been confirmed in experimental studies. These results indicate that miRNAs are extensively involved in pathogenesis of solid cancers and support their function as either dominant or recessive cancer genes. Finding such a signature is important because it shows that many forms of cancer share common genetic pathways that become scrambled as cancer takes hold and spreads. The findings also pave the way for new approaches in diagnosis and treatment. miRNAs themselves may 1 day be used as therapeutics. If miRNAs that are lost are replaced and those that are overly abundant are blocked, it may be possible to prevent some of the very earliest changes that occur in the development of cancer.

Usefulness of miRNAs for the detection, diagnosis, prognosis, and possible treatment of human cancer will depend on carefully designed translational studies. In addition, it will require careful consideration of the best methods for sample collection, miRNA isolation, miRNA quantitation, and data analysis. To facilitate this, there is a need to gain a better understanding of specific miRNA characteristics, such as how targeting of multiple mRNAs by a single miRNA affects data interpretation in biomarker studies and the effect of miRNA isoforms on diagnostic utility. In the therapeutic area, targeting of the correct miRNA sites without affecting miRNA targets of similar sequence will be required.

Diagnostic Value of miRNA in Cancer

Various techniques have been used to detect miRNAs in tumor samples, which has potential clinical applications (Spizzo et al. 2008). miRNAs can be used as tumor predisposition biomarkers in cancer screening programs. miRNAs might have an advantage over gene expression signatures as biomarkers. The number of miRNAs describing a prognostic signature is less than the number of coding genes and, therefore, after the initial phase of defining a distinctive miRNA signature, only a few miRNAs need be tested, by a less expensive technique in a larger set of patients, to validate the previous results and adapt the signature for clinical use. miRNAs that play roles in various steps of metastasis without obvious involvement in tumorigenesis, have also been identified. Understanding how these metastasis-associated miRNA, which we term metastamir, are involved in metastasis will help identify possible biomarkers or targets for the most lethal attribute of cancer: metastasis (Hurst et al. 2009).

Bead-based flow cytometric miRNA expression profiling of miRNAs in samples from multiple human cancers shows distinctive miRNA fingerprints. Generally there is downregulation of miRNAs in tumors compared with normal tissues. The miRNA profiles reflect the developmental lineage and differentiation state of the

tumors and enable successful classification of poorly differentiated tumors whereas mRNA profiles are inaccurate. miRNA profiling may help in the selection of appropriate treatment for cancer. Cancers in which miRNA expression profiling has been shown to be of diagnostic value include chronic lymphocytic leukemia, colorectal cancer, brain tumors, prostate cancer and lung cancer. Further applications are described along with biomarkers involving tumors of different organs.

miRNA expression profiles represent a promising category of disease biomarkers, but population specific genetic variation can affect the prevalence and baseline expression of these miRNAs in diverse populations (Rawlings-Goss et al. 2014). Therefore, variations of genetic and expression level of miRNA among ethnic groups may be contributing to health disparities observed in multiple forms of cancer, and should be considered when assessing clinical applications of miRNA biomarkers.

Biomarkers of Epigenetic Gene Silencing in Cancer

Both genetics and epigenetics regulate gene expression in cancer. Regulation by genetics involves a change in the DNA sequence, whereas epigenetic regulation involves alteration in chromatin structure and methylation of the promoter region. DNA methylation represents an epigenetic means of inheritance without associated DNA sequence alterations. Two major mechanisms that foster epigenetic changes are DNA methylation at cytosine bases within a CpG dinucleotide and histone acetylation. Aberrant CpG island methylation has been associated with changes observed in aging and neoplastic cells. A growing list of genes, including known tumor-suppressor genes, have been shown to have aberrant CpG island methylation in cancer. These epigenetic events act as alternatives to mutations and deletions to disrupt tumor suppressor gene function.

In the past, it was believed that only individual single genes were silenced by methylation. But this is not necessarily the case. Loss of gene expression can occur through long-range epigenetic silencing, with similar implications as loss of heterozygosity in cancer. Non-methylated genes that reside in a particular area near methylated genes may also be silenced. Their physical proximity to the methylated genes affects their ability to function. A fundamental difference between genetic and epigenetic alterations in cancer is the irreversible nature of genetic lesions whereas epigenetic ones are potentially reversible. Cancer therapies, which can reverse DNA methylation, can restore the cell's normal regulation to prevent or stop the progression of cancer. Drugs that promote DNA demethylation are already in clinical trials. Therefore, detection of biomarkers of methylation is important.

Methylation of genes involved in DNA repair and genome integrity (e.g. MGMT, hMLH1, WRN, and FANCF), and cell-cycle checkpoint genes (e.g. CHFR and 14-3-3 sigma, CDK10, and p73), influence the sensitivity to chemotherapeutic drugs, suggesting that DNA methylation could serve as a molecular biomarker for predicting the responsiveness of tumors to chemotherapy (Toyota et al. 2009).

Epigenetic alterations are innovative cancer biomarkers because of stability, frequency, reversibility and accessibility in body fluids, which provides a great potential for development assays to assist in patient management (Costa-Pinheiro et al. 2015). Several studies have identified putative epigenetic cancer biomarkers, some of which have been commercialized. However, large multicenter validation studies are required to foster translation to the clinics.

5-Hydroxymethylcytosine as a Biomarker of Cancer

5-hydroxymethylcytosine (5hmC) is a DNA base created during active DNA demethylation and plays essential roles in gene expression and differentiation. Reduction of hmC levels in DNA is a hallmark of cancers and is a useful biomarker. Various methods used for quantifying and mapping 5hmC include restriction enzyme-based detection, DNA-enrichment strategies, and genome-wide sequencing of 5hmC (Qing et al. 2017). TrueMethyl© (Cambridge Epigenetix), based upon the oxidative bisulfite sequencing, is a state-of-the-art technology for accurate quantification of 5mC and 5hmC in parallel at single-base resolution, which is not possible with traditional bisulfite sequencing.

Carcinoembryonic Antigen

CEA is a cell-surface-bound glycoprotein, which is normally produced during the development of a fetus, but the production of CEA stops before birth, and it usually is not present in the blood of healthy adults. CEA is overexpressed and released by many solid tumors that has an autocrine function in cancer cell survival and differentiation. Soluble CEA released by tumors is present in the circulation of patients with cancer, where it is used as a biomarker of cancer progression. There is evidence that soluble CEA is sufficient to induce proangiogenic endothelial cell behaviors, including adhesion, spreading, proliferation, and migration in vitro and tumor microvascularization in vivo (Bramswig et al. 2013). CEA-induced activation of endothelial cells is dependent on integrin β-3 signals that activate the focal-adhesion kinase and c-Src kinase and their downstream MAP-ERK kinase/extracellular signal regulated kinase and phosphoinositide 3-kinase/Akt effector pathways. While interference with VEGF signaling has no effect on CEA-induced endothelial cell activation, downregulation with the CEA receptor in endothelial cells attenuates CEA-induced signaling and tumor angiogenesis. Clinical correlation shows that tumor microvascularization is higher in patients with CRC exhibiting higher serum levels of soluble CEA indicating a novel function for soluble CEA in tumor angiogenesis.

Circulating Cancer Biomarkers

As an alternative to cancer tissue biopsy, biomarkers can be identified on fluid biopsy of body fluids, mostly circulating plasma. Three main types of circulating biomarkers will be discussed in this section: circulating tumor cells, circulating nucleic acids, and exosomes or microvesicles.

Circulating Tumor Cells as Cancer Biomarkers

Circulating tumour cells (CTCs) are potential prognostic biomarkers of cancer and a reliable mean to predict metastasis development. The presence of CTCs as a prerequisite for development of distant metastasis is known since the nineteenth century. In clinical trials, CTC counts have been used as biomarkers for prognostic stratification and evaluation of disease response during therapy. The number of CTCs could be useful for prognosis in early stage of disease, for identification of patients requiring adjuvant therapy, or during follow-up to detect relapses. Other clinical applications are based on the molecular and genetic characterization of CTCs. These applications have been shown to be valuable in several clinical trials of lung cancer and breast cancer. The FDA has approved CellSearch® system for CTC enumeration (Veridex/Johnson & Johnson). However, CTC enumeration may not provide a comprehensive picture of a patient's status and the search continues for other parameters, e.g. sequencing for further information.

Circulating Nucleic Acids as Potential Biomarkers of Cancer

Both DNA and miRNA (see later in this chapter) have been studied as cancer biomarkers. Circulating cell-free DNA (ccf-DNA) levels have been studied in plasma and serum samples as biomarkers of cancer. An elevated level of ccf-DNA has been detected in the circulation of cancer patients in comparison with healthy controls (Kohler et al. 2011). Since ccf-DNA in cancer patients often bears similar genetic and epigenetic features to the related tumor DNA, there is evidence that some of the ccf-DNA originates from tumor tissue. This, and the fact that ccf-DNA can easily be isolated from the circulation and other body fluids of patients, makes it a promising candidate as a noninvasive biomarker of cancer. ccf-DNA may also represent an important source of biomarkers at several steps of carcinogenesis, including early detection of preneoplastic lesions and monitoring of cancer. Moreover, levels of plasma DNA could be tested as a potential intermediate biomarker of the efficacy of intervention. It is possible to develop a simple cost-effective blood test, with high sensitivity and specificity that has potential for screening high-risk individuals, for prognostic purposes and to be used as intermediate end-points of efficacy in chemoprevention and therapeutic trials (Catarino et al. 2012).

Circulating Exosomes and Microvesicles as Biomarkers of Cancer

Exosomes are small vesicles (50–100 nm) secreted by almost all tissues; they represent their tissue of origin. Exosomes although used synonymously are distinguished from microvesicles which are heterogeneous in size (50–1500 nm) and shed directly from the budding of the plasma membrane. Tumor cells release an abundance of exosomes and microvesicles containing a selected set of proteins and RNAs. Exosomes are valuable sources for biomarkers due to selective cargo loading and resemblance to their parental cells. It has been shown that tumor microvesicles and exosomes also carry DNA, which reflects the genetic status of the tumor, including amplification of the oncogene c-Myc (Balaj et al. 2011). This study also found amplified c-Myc in serum microvesicles from tumor-bearing mice. Further, the authors found remarkably high levels of retrotransposon RNA transcripts, especially for some human endogenous retroviruses, such as LINE-1 and Alu retrotransposon elements, in tumor microvesicles and these transposable elements could be transferred to normal cells. These findings expand the nucleic acid content of tumor microvesicles to include: elevated levels of specific coding and non-coding RNA and DNA, mutated and amplified oncogene sequences and transposable elements. Thus, tumor microvesicles or exosomes contain a repertoire of genetic information available for horizontal gene transfer and potential use as biomarkers for cancer in circulating blood.

Cancer-derived exosomes contribute to cancer progression by enhancement of intercellular transfer of cargo that contains proteins, lipids and nucleic acids within the tumor microenvironment. This reflects the altered state of original cancers, making exosomes as biomarkers for early detection, diagnosis and prognosis with higher sensitivity and specificity compared to conventional biopsy or other liquid biopsy biomarkers (Soung et al. 2017). The value of exosomes as biomarkers for breast cancer, prostate cancer, pancreatic cancer, and glioblastoma multiforme is described in sections dealing with these cancers.

Circulating miRNAs for Cancer Detection

miRNAs are present in human plasma in a remarkably stable form that is protected from endogenous RNase activity. Measurement of tumor-derived miRNAs in serum or plasma is an important approach for the blood-based detection of human cancer. Locked nucleic acid (LNA) is a conformational RNA analog that binds to RNA with unprecedented affinity and specificity, making it well suited for miRNA detection and analysis for cancer diagnostics (Stenvang et al. 2008).

DNA Repair Biomarkers

All solid cancers have multiple pathways. Most chemotherapies, and all radiation therapy, work by damaging DNA, which can usually enable tumor cells to survive DNA damage induced by chemotherapeutic treatments. Therefore, inhibitors of

specific DNA repair pathways might prove effective when used in combination with DNA-damaging chemotherapeutic drugs. In addition, alterations in DNA repair pathways that arise during tumor development can make some cancer cells reliant on a reduced set of DNA repair pathways for survival. There is evidence that drugs that inhibit one of these pathways in such tumors could prove useful as single-agent therapies, with the potential advantage that this approach could be selective for tumor cells and have fewer side effects (Helleday et al. 2008). Proteomic biomarkers in each of the pathways differ according to the type of cancer but they overlap. Since most available cancer therapeutics work by inducing DNA damage, which causes cell death, monitoring specific DNA repair biomarkers may indicate whether the therapeutic is producing tumor cell death. DNA repair biomarkers could enable physicians to monitor treatment effectiveness from solid tumor samples. On-Q-ity is developing a cancer diagnostic platform based on DNA repair biomarkers that can be used to predict patient's response to treatment and will be useful for personalized management of cancer.

HER3 as Biomarker of Cancer

HER3, a co-receptor of HER2, plays an important and dominant role in the functionality and transformation of HER-mediated pathways. Understanding the role of HER3 in oncogenesis as well as its place as a target for anticancer therapy is an ongoing area of research. Determination of biomarkers for clinical benefit from agents targeting HER3 is an essential component of translating basic science into real-world effective anticancer therapies, with the aim of ensuring the patients most likely to benefit from such treatments can be identified. HER2 and HER3 can be targeted by monoclonal antibodies and the potential for HER3 mRNA levels to predict treatment outcome in ovarian cancer (Amler 2010). An understanding of the value of HER3 mRNA as a biomarker is important for clinical benefit of the HER2-HER3 dimerization inhibitor pertuzumab. In conclusion, HER3 mRNA levels may be a biomarker for active ligand-induced HER2-HER3 signaling, with low HER3 mRNA levels correlated with clinical benefit from the HER2-HER3 dimerization inhibitor.

Immunologic and Inflammation Biomarkers of Cancer

The immune system and inflammation are implicated in the pathogenesis of cancer. Improvements in techniques have enabled demonstration of tumor infiltrating lymphocytes (TILs) in different tumor compartments. TILs are associated with improved prognosis in cancer. As immunologic biomarkers, TILs have an important role in determining outcomes of clinical interventions as intermediate end-points. TILs are important as biomarkers for the development of cancer immunotherapies.

Several prospective studies have investigated the link of biomarkers of inflammation with cancer incidence and mortality. A prospective cohort study enrolled postmenopausal women who were free of cancer at baseline in Women's Health Initiative (Margolis et al. 2007). The main outcome measures were incident invasive breast, CRC, endometrial, and lung cancer. In multivariate models, there was a graded association of WBC count with incidence of all 4 types of cancer. Thus postmenopausal women with higher WBC counts have a higher risk of incident invasive breast, CRC, endometrial and lung cancer, as well as a higher risk overall cancer mortality.

Metastatic Cancer Biomarkers

Biomarkers of metastases of various cancers have been investigated. Examples are given along with specific cancers. Examples are as follows:

CUB domain-containing protein 1 (CDCP1) is a transmembrane protein that is highly expressed in stem cells and frequently overexpressed and tyrosine-phosphorylated in cancer. CDCP1 promotes cancer cell metastasis. A study has shown that hypoxia induces CDCP1 expression and tyrosine phosphorylation in hypoxia-inducible factor (HIF)-2α–, but not HIF-1α–, dependent fashion (Emerling et al. 2013). shRNA knockdown of CDCP1 impairs cancer cell migration under hypoxic conditions, whereas overexpression of HIF-2α promotes the growth of tumor xenografts in association with enhanced CDCP1 expression and tyrosine phosphorylation. IHC analysis of tissue microarray samples from tumors of patients with clear cell renal cell carcinoma shows that increased CDCP1 expression correlates with decreased overall survival. Together, these data support a critical role for CDCP1 as a unique HIF-2α target gene involved in the regulation of cancer metastasis, and suggest that CDCP1 is a biomarker and potential therapeutic target for metastatic cancers.

Mena protein potentiates and modulates cellular migration and is found in the developing embryo where it plays an important role in the developing nervous system among other functions. It facilitates and organizes formation, extension and navigation of growing nerve fibers through tissue to link with other neurons, forming the proper circuits needed for a functional nervous system. However, in metastatic cancer cells, high levels of the Mena protein accumulate and influence a number of intracellular signaling programs. Mena facilitates a process whereby tumor cells send out a well-organized protuberance that invades surrounding tissue and pulls the remainder of the cell behind it. Mena modulates the strength and direction of this invasive process and steers the migrating cancer cell in the direction of blood vessels through its ability to modulate the metastatic cell's response to chemical signals that attract it to blood vessels. Mena is present in cancer cells in several isoforms that are similar but slightly different in structure. Despite similarity in structure, protein isoforms differ considerably in their influence on cells. MetaStat has identified the most dangerous isoform of Mena named MenaINV (Mena

invasive). Mena11A, on the other hand, is the Mena isoform that seems to exert a much more positive influence on the cell's behavior, reducing the ability of cells to break away from the tumor and invade and migrate toward blood vessels. Metastat's key discovery is that it can predict the metastatic potential of a cancer cell by measuring the relative levels of MenaINV and Mena11A. As the relative levels of MenaINV rise and Mena11A fall the cancer cell transitions to a more metastatic shape and behavior. These metastasis promoting behavior changes include increased migratory behavior, changes in shape, loss of adhesion to neighboring cells, and up to 100-fold greater sensitivity to the chemical attractant that lures metastatic cells to blood vessels.

In 2013, MetaStat signed two licensing agreements – one with the Massachusetts Institute of Technology and the other with Montefiore Medical Center – for the use of alternatively spliced mRNA and Mena protein isoform biomarkers for diagnosis, prognosis, as well as treatment of metastases of solid epithelial cancers. This platform directly links a therapeutic to its companion diagnostic based on the detection and targeting of alternatively spliced oncogenes, which drive tumor progression and resistance, thereby offering a unique opportunity for personalized treatment of cancer.

Molecular Diagnostic Techniques for Cancer

Molecular diagnostic techniques applicable to the diagnosis of cancer are shown in Table 13.3 and are described briefly in the following sections.

Technologies for Detection of Cancer Biomarkers

Several technologies are in use for detection of individual cancer biomarkers. A molecular diagnostic assay is essentially a combination of five steps:

- Sample liquefaction
- Cell lysis
- DNA/RNA extraction
- Target amplification and detection
- Data analysis and reporting

Biocartis' molecular diagnostics platform Idylla is used as a navigation system through the world of oncology. It combines proprietary and versatile sample preparation technology with state-of-the-art multiplexed real-time PCR amplification and detection. Up to 30 targets can be detected in one single cartridge. The biomarkers can be SNPs, mutations, translocations or changes in RNA expression profiles. This makes the system suitable for a very broad range of assays and sample types for cancer biomarker detection.

Table 13.3 A classification of molecular diagnostic methods in cancer

Genomic technologies
Assays for determining genetic susceptibility to cancer
DNA tags for finding genes expressed in cancer
Genome analysis at molecular level
Restriction fragment length polymorphism (RFLP)
Loss of heterozygosity (LOH)
Nonradioactive mutation screening
Sequencing-based approaches
Single-strand conformation polymorphism (SSCP)
Polymerase chain reaction (PCR)
Reverse transcriptase (RT)-PCR
Real-time quantitative PCR
COLD-PCR
Gene expression profiling
cDNA microarrays to analyze gene expression
Human aspartyl (asparaginyl) hydroxylase (HAAH) gene
Serial analysis of gene expression (SAGE)
Cancer cytogenetics
Array comparative genomic hybridization (aCGH)
Digital karyotyping
Fluorescent in situ hybridization (FISH)
Expression profiling of tumor cells sorted by flow cytometry
Immunohistochemistry
Detection of DNA methylation
In vivo molecular diagnosis of cancer by positron emission tomography
Detection of tumor cells in body fluids
Detection of tumor cells in circulating blood
Detection of tumor cells in urine
Detection of tumor cells in cerebrospinal fluid
Assays based on proteins and enzymes
P53 sequencing and functional assays
Measurement of telomerase activity
Prognostic assay based on survivin (inhibitor of apoptosis protein)
Molecular histopathology of cancer
Proteomic technologies for discovery of cancer biomarkers
Antibody microarrays
Aptamer-based technology for protein signatures of cancer cells
Detection of circulating nucleosomes in serum
Detection of oncoproteins
eTag assay system for cancer biomarkers
Gel electrophoresis
Laser capture microdissection (LCM)
Modifications of mass spectrometry (MS): SELDI TOF MS, desorption electrospray ionization
Phage display
Proteomic analysis of cancer cell mitochondria
ProteinChip technology
Application of nanobiotechnology for discovery of cancer biomarkers

Genomic Technologies for Cancer Biomarkers

Biomarkers of PTEN Tumor Suppressor Gene Status

PTEN is an important tumor-suppressor gene associated with many cancers. Through expression profiling of glioblastoma tissue samples and prostate cancer xenografts, a molecular signature for loss of the PTEN tumor suppressor has been identified in glioblastoma and prostate tumors (Mehrian-Shai et al. 2007). The PTEN signature consists of a minimum of nine genes, several of which are involved in various pathways already implicated in tumor formation. Among these signature genes, the most significant is an increase in insulin growth factor-binding protein 2 (IGFBP-2) mRNA. The link between IGFBP-2 and PTEN is of particular interest because elevated serum IGFBP-2 levels have been reported in patients with prostate and brain tumors. IGFBP-2 expression is negatively regulated by PTEN and positively regulated by phosphatidylinositol 3-kinase (PI3K) and Akt activation. In addition, Akt-driven transformation is impaired in IGFBP2-/- mouse embryo fibroblasts, implicating a functional role for IGFBP-2 in PTEN signaling. Collectively, these studies establish that PTEN and IGFBP-2 expression are inversely correlated in human brain and prostate cancers and implicate serum IGFBP-2 levels as a potential serum biomarker of PTEN status and PI3K Akt pathway activation in cancer patients.

Cold-PCR

COLD-PCR (co-amplification at lower denaturation temperature-PCR is a novel form of PCR that amplifies minority alleles selectively from mixtures of wild-type and mutation-containing sequences irrespective of the mutation type or position on the sequence (Li and Makrigiorgos 2009). For clinically relevant micro-deletions, COLD-PCR enabled exclusive amplification and isolation of the mutants. It enriches mutations in cancer samples where normal DNA predominates. The range of enrichment demonstrated to date varies from 3 to 100-fold. Its effectiveness has been demonstrated in enriching for mutations in cancer-related genes in samples where DNA sequencing cannot detect very low concentrations of somatic DNA mutations. COLD-PCR is expected to have diverse applications in the fields of biomarker identification and tracing, genomic instability, infectious diseases, DNA methylation testing and prenatal identification of fetal alleles in maternal blood.

ddPCR for Detection of Cancer Biomarkers in Cell Free Plasma DNA

Tumor genotyping using cell free plasma DNA (cfDNA) has potential for noninvasive assessment of tumor biology, yet many existing assays are cumbersome and may give false positive results. Noninvasive genotyping of cfDNA using

droplet digital PCR (ddPCR) could enable effective translation into a clinical diagnostic. Serial use of ddPCR in EGFR-mutant lung cancer patients in a prospective trial of erlotinib allowed assessment of response and resistance, including detection of resistance mutations up to 16 w prior to radiographic progression (Oxnard et al. 2014).

Comparison of miRNA quantification by ddPCR and real-time PCR has revealed greater precision and improved day-to-day reproducibility (by a factor of seven) of ddPCR but with comparable sensitivity. Applied ddPCR to serum miRNA biomarker analysis, this translated to superior diagnostic performance for identifying individuals with cancer (Hindson et al. 2013). This approach was tested in advanced prostate cancer and age-matched male controls to measure the abundance of miR-141, which has been shown to be a biomarker for advanced prostate cancer. Compared to analysis of samples by qPCR ddPCR improved day-to-day reproducibility 7-fold. ddPCR enables accurate follow-up of serum miRNA biomarker concentrations over time during the course of treatment, which is nearly impossible to achieve with real-time PCR.

Digital Karyotyping for Cancer Biomarkers

Digital karyotyping has been developed for a genome-wide analysis of DNA copy number alterations at high resolution. The principle of this approach is similar to the SAGE method, which is based on the isolation and enumeration of short sequence tags. However, the sequence tags in digital karyotyping are obtained from genomic DNA rather than from mRNA, and they are isolated by different methods. These tags contain sufficient information that allows assigning the tag sequences to their corresponding genomic loci from which they are derived. The number of each unique tag along each chromosome can be used to quantitatively evaluate DNA content in tumor samples. Digital karyotyping can identify all the known chromosomal alterations and reveal several distinct genetic alterations that are not shown by other methods. Several undiscovered copy number alterations exist in cancer genomes and many of these could be detected through digital karyotyping. Digital karyotyping has also been applied in identifying specific gene amplification in colorectal cancer patients that is associated with resistance to chemotherapy with 5-fluorouracil. Identification of previously uncharacterized amplified genes in cancer provides new biomarkers for diagnosis and as well as targets for therapy in cancer.

Genome Analysis at the Molecular Level

When carried out at the molecular level, genome analysis can provide high resolution of the tumor DNA and the patient's constitutional DNA, enabling the subsequent detection of allelic imbalances by Southern blotting. This approach uses naturally occurring polymorphisms to obtain the distribution of the recognition sites

for specific restriction enzymes (restriction fragment length polymorphism). Because this analysis utilizes biochemically extracted DNA from fresh or frozen tumor tissue, no information is obtained at the single cell level. In addition, contamination of the tumor DNA with that from admixed non-neoplastic cells may create interpretational problems. The application of DNA polymorphism to the study of human cancers has, however, provided important clues as to the biological significance of imbalances in chromosomal material, which are frequently observed in cytogenetic studies of tumors. Allelotyping of the individual tumors requires considerable labor and would take several months with the current set of histopathological classification systems.

Gel electrophoresis is a useful method in cancer molecular diagnostics. It is at least 10 times as sensitive as Southern blot assay. It can readily determine clonality in the primary diagnosis of a neoplasm. CGH can accurately identify multiple or missing DNA sequences along the entire chromosome with the aid of "biochips."

Cost-effective methods for sequencing the whole genome in tissue specimens has led to the identification of numerous molecular abnormalities in cancers – mutations, amplifications, insertions and deletions of genes – as well as patterns of differential gene expression, i.e., overexpression of growth factors and underexpression of tumor suppressor genes. These abnormalities can be translated into assays to be used for clinical decision making. A review discusses the effects of these variables on assays of tissue-based biomarkers, classified by macromolecule – DNA, RNA (including miRNA, mRNA, lcRNA, protein, and phosphoprotein (True 2014). Since the majority of clinically applicable biomarkers are proteins detected by IHC, focus is on protein biomarkers. The appropriateness of an assay for clinical application is an important issue. Reference is made to publicly available guidelines to improve on biomarker development in general and requirements for clinical use in particular. Strategic goals have been formulated to improve the quality of biomarker reporting, including issues of analyte quality, experimental detail, assay efficiency and precision, and assay appropriateness.

KRAS as a Biomarker of Cancer

KRAS mutation is the most common in various human cancers. And has emerged as an important predictive biomarker in some of the common cancers such as CRC and NSCLC (Kriegshäuser et al. 2010). KRAS may prove useful as a diagnostic biomarker to screen for early pancreatic cancer, and quantitative KRAS mutation analysis may be useful for distinguishing pancreatic cancer from other conditions such as chronic pancreatitis. It may serve as a biomarker for predicting response to EGFR-targeted therapy, and may also serve as a diagnostic and predictive biomarker for evolving therapies directed against mutant RAS proteins. However, multiplex approaches including other biomarkers should facilitate the identification of patients likely to respond to such therapies.

LigAmp for Detection of Gene Mutations in Cancer

LigAmp is a molecular tool designed to pinpoint DNA mutations among thousands of cells. The test works by creating a molecular "magnet" with an affinity for the point mutation. If the mutation is found, the magnet binds to it and inserts a bacterial gene. The bacterial gene serves as a red flag and produces a fluorescent color visible to powerful computer programs. Preliminary studies in a small number of cell lines and body fluids show the ultra-sensitive test may help detect microscopic cancer and HIV drug resistance. LigAmp essentially filters background 'noise' caused by normal cells and reveals specific mutations. It can form the basis of sensitive tests to locate mutations and identify cancer in patients at high-risk for the disease. Such tests could even help detect a recurrence of cancer by monitoring whether the number of mutations rises above a predetermined threshold value. Single mutations in colon cancer cells and mutations of the KRAS2 gene were detected in duct fluid samples from pancreatic cancer patients, which also corresponded to mutations found in their tumors.

Mitochondrial DNA as a Cancer Biomarker

Circulating cell-free (ccf) mtDNA in blood is a non-invasive diagnostic and prognostic biomarker for many forms of solid tumors. Plasma or serum ccf mtDNA levels are significantly different between cancer patients and healthy individuals (Yu 2012). Leukocyte mtDNA content is an independent prognostic biomarker complementing TNM (tumor-nodes-metastasis) stage and associated with immunosuppression in CRC patients (Qu et al. 2015). Circulating levels of mtDNA and IL8, as measured by quatitative real-time PCR, constitute a potential biomarker for the early detection of gastric cancer (Fernandes et al. 2014). Additionally, leukocyte mtDNA content might serve as a potential biomarker to select CRC patients who will benefit from adjuvant chemotherapy. Heteroplasmic and homoplasmic sequence variants occur in the mitochondrial genome in tumors of patients. These changes can be identified by sequencing mitochondrial DNA (mtDNA) obtained from tumors and blood from the same individual. Rapid and high-throughput sequencing has been used for the detection of sequence variants in mtDNA. Relatively simple diagnostic tests for detecting mtDNA mutations, involving mitochondrial microarrays and real-time PCR, have predictive potential for cancer detection and prognosis. mtDNA DNA alterations (mutations, deletions and instability) are emerging as biomarkers for detecting a variety of cancers in tissue samples and biofluids which can be included in population screening studies. Using a oligonucleotide microarray (MitoChip) for rapid sequencing of the entire mitochondrial genome, somatic mtDNA alterations were observed in preneoplastic lesions even in the absence of histopathologic evidence of dysplasia. A 3.4-kb mitochondrial genome deletion (3.4 mtdelta) was reported to be useful for molecular definition of benign, malignant, and proximal to malignant prostate needle biopsy specimens (Maki et al. 2008). These findings support the rationale for exploring the mitochondrial genome as a biomarker for the early diagnosis of cancer.

Next Generation Squencing for Detection of Cancer Biomarkers

Next generation sequencing (NGS)-based approaches for associating genomic rearrangements or structural variants with RNA expression profiles are useful for detection of biomarkers of cancer. Mutations in DNA sequence are transcribed to mRNA and ultimately translate into a functional protein. Thus mRNA may regulate processes such as alternative splicing and RNA editing and a variety of cellular functions.

The most widely used method to analyze global patterns of RNA expression, the DNA microarray, offers limited sensitivity, requires greater sample input and is unable to detect novel RNAs. Alternatively, a sequencing-based approach to RNA expression analysis enables researchers to detect low levels of expression invisible on microarray platforms, and perform a hypothesis-neutral analysis of gene expression profiles, enabling the detection of all known and novel RNAs present in biological samples, with no bias toward known RNA molecules as with probe-based array technologies. Sequencing-based RNA expression analysis has enabled the researchers to establish directionality of expressed transcripts which is significant because DNA is transcribed in two different directions. Establishing directionality of expressed transcripts allows researchers to more easily determine which RNA transcripts, are coding and non-coding. Non-coding RNAs play an increasingly important role in regulating biological processes involved in cancer differentiation and development. Enormous amounts of data are being generated by NGS and microarray analysis. With further advances, sensitive and specific miRNAs/mRNA signatures may be identified and become routinely used for cancer detection, as well as diagnosis and prognosis in clinical trials (Li et al. 2015).

In 2017, QIAGEN licensed genetic biomarkers to assess microsatellite instability (MSI) and mismatch repair (MMR) in all cancer sample and cell types from the Johns Hopkins University for developing NGS-based tests to assess MSI and MMR status. Bothe MSI and MMR, along with tumor mutation burden, are important for identifying cancer patients who could benefit from certain types of immune therapies. In 2017, the FDA approved an immune therapy to treat advanced solid tumors with MSI and MMR deficiencies, marking the first time that the FDA has cleared a cancer drug for use not tied to the site of a tumor but to a biomarker.

Telomerase as a Biomarker of Cancer

Telomeres, the tandem-repeated hexamers at the termini of mammalian chromosomes, form protective complexes in association with specific proteins that together with telomerase, a specialized telomere-synthesizing enzyme, regulate telomere length. Telomere shortening is associated with cellular senescence and is implicated in tumorigenesis and cancer. Since the hallmark report of the PCR-based telomeric repeat amplification protocol in 1994, there has been a flurry of investigations of telomerase activity on normal, benign, premalignant and cancerous samples representative of the various stages of tumorigenesis. Discovery of mutations within the core promoter of the telomerase reverse transcriptase (TERT) gene that create de novo binding sites for transcription factors provided a mechanism for

cancer-specific telomerase reactivation. The TERT promoter mutations occur mainly in tumors from tissues with low rates of self-renewal. In melanoma, glioma, hepatocellular carcinoma, urothelial carcinoma and others, the promoter mutations have been shown to define subsets of patients with adverse disease outcomes, associate with increased transcription of TERT, telomerase reactivation and affect telomere length (Heidenreich and Kumar 2017). Thus, the TERT promoter mutations have potential as biomarkers as well as future therapeutic targets.

Despite the wide variety of studies on the potential use of telomerase as a cancer biomarker, the variability of reported telomerase activity and the lack of a transferable detection method have prevented it from becoming a routine clinical application. Real-time PCR is a clinically transferable method and the advancement of real-time measurements of telomerase will facilitate moving telomerase activity and technologies towards a biomarker for clinical validation. It is expected that telomerase will be integrated into the initial detection and follow-up monitoring of cancer patients.

Tissue Microarrays for Study of Cancer Biomarkers

The use of tissue microarrays (TMAs) as a strategy to validate and characterize candidate biomarkers in cancer is gaining widespread acceptance as an alternative to more traditional immunohistochemical (IHC) methods. Less widely appreciated, is that TMAs also facilitate a rapid assessment of biomarker expression in larger cohorts of tissue and are thereby amenable to the development of statistical models based on structured IHC information.

Manual interpretation of IHC is a subjective, time-consuming and variable process, with an inherent intra-observer and inter-observer variability. Automated image analysis systems offer the possibility of developing rapid, uniform indicators of IHC staining. A study has described the development of a novel approach for automatically quantifying oestrogen receptor (ER) and progesterone receptor (PR) protein expression assessed by IHC in primary breast cancer (Rexhepaj et al. 2008). A fully automated nuclear algorithm was developed to discriminate tumor from normal tissue and to quantify ER and PR expression in both cohorts. Random forest clustering was employed to identify optimum thresholds for survival analysis. The data on the automated quantification of the ER and the PR in primary breast tumors provides a useful tool for the quantification of biomarkers on tissue specimens, as well as for objective identification of appropriate cutoff thresholds for biomarker positivity. It also offers the potential to identify proteins with a homogeneous pattern of expression.

Use of digital pathology and image analysis is expanding largely due to the implementation of whole slide scanning, advances in software and computer processing capacity and the increasing importance of tissue-based research for biomarker discovery and personalized medicine. Digital pathology and image analysis

applications include tissue architecture analysis with emphasis on IHC and fluorescence analysis of tissue biomarkers. They play important roles in drug/companion diagnostic development pipeline including biobanking, molecular pathology, tissue microarray analysis, molecular profiling of tissue. Digital image analysis data should be integrated with epidemiological, clinical and genomic data in order to fully understand the relationship between genotype and phenotype and to drive discovery and the delivery of personalized medicine (Hamilton et al. 2014).

Molecular Fingerprinting of Cancer

Current cancer diagnostics are largely based either on imaging or the analyses of several biomarkers. However these methods are not precise, as they do not reveal the molecular structure of the tumor cells and, in most cases, do not allow for early detection. Several companies and research groups are developing proteomics-based diagnostics that identify cancer related protein and peptide patterns in the blood serum. Although these diagnostics, when fully developed, have the potential to be precise and suitable for early detection, they will not provide the molecular signature or fingerprint of individual tumors. Also under development are molecular diagnostic techniques based on genomic and proteomic analyses of tumor samples. While this method is precise and results in a molecular characterization of cancer cells, it is inadequate for early detection, mass screening and monitoring because it requires a biopsy from the tumor. Unfortunately for patients, by the time the tumor is detected, it is typically too large and too advanced for effective treatment.

To overcome some of these limitations, a 'molecular fingerprinting'-based blood tests have been developed for deciphering the molecular structure of individual tumors. The advantages of these tests are:

- Early/accurate detection with fewer false positives and false negatives
- Minimally invasive testing of blood or body fluids such as urine, sputum and saliva
- For prediction of optimal treatment for an individual specific patient
- For help in designing specific diagnostic methods and drugs to treat cancer

Proteomic Technologies for Detecting Biomarkers of Cancer

Proteins may be actively secreted or released by the tumor cells as a result of necrosis or apoptosis and released into the circulation. They change the protein profile. The difference in signal intensities may be detected by comparison with sera from normal individuals. A scheme for the role of proteomics in discovery of cancer biomarkers is shown in Fig. 13.1. Some of the technologies are described briefly in the following sections.

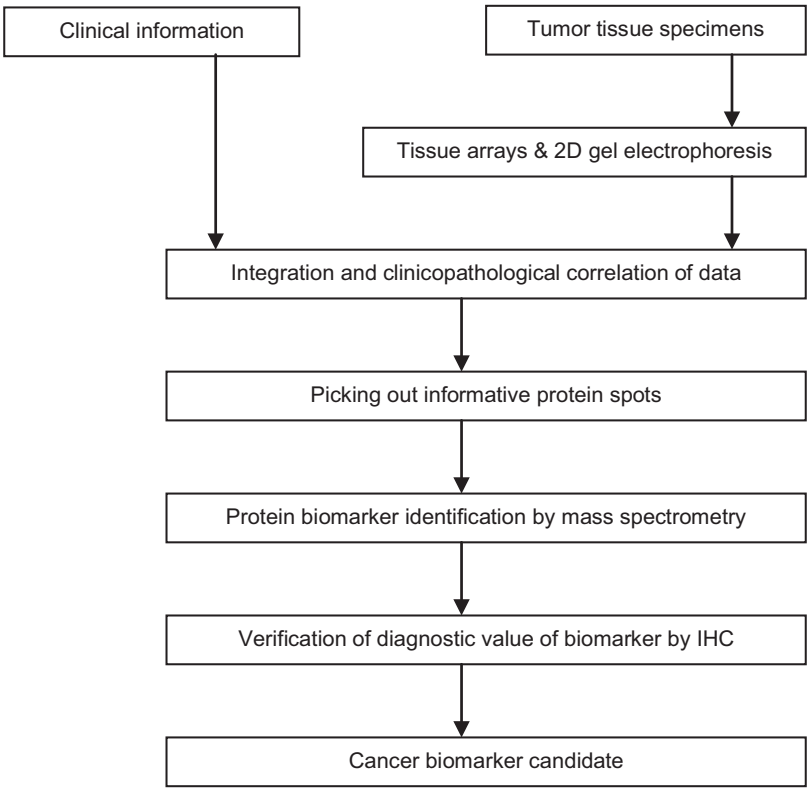

Fig. 13.1 Role of proteomics in the discovery of cancer biomarkers. (© Jain PharmaBiotech)

2D PAGE

2D PAGE followed by protein identification using MS has been the primary technique for biomarker discovery in conventional proteomic analyses. This technique is uniquely suited for direct comparisons of protein expression and has been used to identify proteins that are differentially expressed between normal and tumor tissues in various cancers, such as liver, bladder, lung, oesophageal, prostate and breast.

Advantages of 2D PAGE for discovery of biomarkers are: (1) it is a tested and reliable method; (2) it can identify markers directly; and (3) it is reproducible and quantitative when combined with fluorescent dyes. Disadvantages of the use of 2D-PAGE for this purpose are: (i) it requires a large amount of protein as starting material making it unreliable for detecting and identifying low-abundance proteins; (ii) early-stage cancers are often small and contamination from surrounding stromal tissue that is present in the specimen can confound the detection of tumor-specific markers; and (iii) low sensitivity. The sensitivity can be improved by laser capture microdissection.

Antibody-Based Detection of Protein Biomarkers

T regulatory cells (Treg) expressing the FOXP3 transcription factor maintain immunological self-tolerance and enable tumor cells to escape immunosurveillance. FOXP3 is a biomarker in human malignancies and antibodies used to detect FOXP3 protein expression. FOXP3 can be used in routine clinical practise to provide both diagnostic and prognostic information, but the methods and reagents used to detect FOXP3 have a significant effect on the robustness of results (Bignone and Banham 2008).

The use of antibody microarrays continues to grow rapidly due to the recent advances in proteomics and automation, and the opportunity this combination creates for high throughput multiplexed analysis of protein biomarkers. However, a primary limitation of this technology is the lack of PCR-like amplification methods for proteins. Therefore, to realize the full potential of array-based protein biomarker screening it is necessary to construct assays that can detect and quantify protein biomarkers with very high sensitivity, in the femtomolar range, and from limited sample quantities. Antibody ultramicroarrays for the detection of IL-6 and PSA, a widely used biomarker for prostate cancer screening, are available. They have a high specificity and sensitivity with detection levels using purified proteins in the attomole range. These ultramicroarrays enable detection of PSA secreted from as few as 4 cells in 24 h. This strategy should enable proteomic analysis of materials that are available in very limited quantities such as those collected by LCM.

Aptamer-Based Molecular Probes for Cancer Biomarker Discovery

Aptamers (derived from the Latin word ‘aptus’ = fitting) are short DNA or RNA oligomers, which can bind to a given ligand with high affinity and specificity due to their particular 3D structure and thereby antagonize the biological function of the ligand. Aptamers are used to detect the protein signatures of cells. This is based on the tendency of short DNA molecules – oligonucleotides – to fold into shapes that bind to specific proteins. The aptamer hugs its protein in the same way as an antibody embraces a specific antigen. The technology can be used with biochips. Aptamer technology may provide a method for monitoring protein changes in the blood that can echo the onset of carcinogenesis, for example in women with genetic risk of breast cancer associated with BRCA1 dysfunction.

Cell-based aptamer selection has been developed to exploit the differences at the molecular level between any two types of cells for the identification of molecular signatures on the surface of targeted cells. Selected aptamers can bind to target cells with an equilibrium dissociation constant (Kd) in the nanomolar-to-picomolar range. The cell-based selection process is simple, fast, straightforward, and reproducible, and, most importantly, can be done without prior knowledge of target molecules. The selected aptamers can specifically recognize target leukemia cells mixed with normal human bone marrow aspirates and can also identify cancer cells closely

related to the target cell line in real clinical specimens. Cell-based aptamer selection holds a great promise in developing specific molecular probes for cancer diagnosis and cancer biomarker discovery.

Biomarkers of Protein-Drug Interactions in Cancer

The challenge of determining drug mechanism requires an understanding of the affinity of the drug for all potential targets. Drug target engagement can be assessed by means of a cellular thermal shift assay (CETSA) based on ligand-induced changes in protein thermal stability. A study combined CETSA with quantitative MS to study the effect of drugs on the thermal profile of a cellular proteome comprising >7000 proteins, which enabled the monitoring of drug targets and downstream effectors (Savitski et al. 2014).

Thermal proteome profiling (TPP) was performed on human cells by heating intact cells and observed marked differences in melting properties between the two settings, with a trend toward increased protein stability in cell extract. ATP-binding proteins showed a significant trend toward increased stability in intact cells, suggesting stabilization by the endogenous ligand. This was confirmed with the addition of ATP to cell extract, which resulted in increased stability for this protein group. The ability of TPP to identify target binding was validated by using the broad-specificity inhibitors such as staurosporine, which induce shifts in the melting temperatures of many kinase targets and also affect the thermal profiles of other proteins, including regulatory subunits of kinase complexes. This study identified the heme biosynthesis enzyme ferrochelatase (FECH) as an off-target of several kinase inhibitors and showed that the drug vemurafenib reaches full target occupancy of its cognate target BRAF and the off-target FECH within a narrow concentration window. FECH deficiency is genetically linked to protoporphyria, suggesting that the photosensitivity induced by vemurafenib and other drugs is mediated by FECH. Drug treatment of live cells affected not only direct target proteins but also downstream effectors. The ABL inhibitor dasatinib induced thermal shifts in several proteins downstream of BCR-ABL, including CRKL, and at concentrations in good agreement with the effect on cell growth. Thermal profiling of cellular proteomes enables the differential assessment of protein ligand binding and other protein modifications, providing an unbiased measure of drug-target occupancy for multiple targets and facilitating the identification of biomarkers for drug efficacy and toxicity.

Cancer Immunomics to Identify Autoantibody Signatures

Cancer immunomics has been used to identify autoantibody signatures produced in response to the presence of either breast or colorectal cancer. SERological proteome analysis (SERPA) is performed by 2D GE, immunoblotting, image analysis, and MS (Hardouin et al. 2007). Alternatively, to identify the antigens recognized by the

autoantibodies of cancer patients, an approach has been developed that combines 2D immunoaffinity chromatography, enzymatic digestion of the isolated antigens, nano flow separation of the resulting peptides, and identification: MAPPing (multiple affinity protein profiling). By these approaches both proteins recognized by autoantibodies independently of a cancer status are identified as well as a limited number of proteins reacting preferentially with cancer sera.

Desorption Electrospray Ionization for Detection of Cancer Biomarkers

A modified mass spectrometry (MS) technique, desorption electrospray ionization (DESI), involves aiming a fine water mist at a surface with a pencil-sized tube that also sucks up the fluid after the droplets have mixed with the material in the sample. Whereas ordinary MS is both time- and labor-intensive, DESI not only is portable, but they can also determine the chemical composition of an unprepared sample within five seconds. Thus DESI can detect chemical signature of cancer in liver tissue. The technique can tell the difference between diseased and non-diseased regions of tissue samples within a few seconds. Another advantage of DESI is that it can detect lipid biomarkers whereas conventionl MS is good at detection protein biomarkers. Cancerous regions possess higher levels of certain lipid molecules, which could indicate a significant relationship between lipids and tumor proliferation. The devices might one day prove useful in helping surgeons ensure that all of the tumor is destroyed before a patient leaves surgery and also to identify other potential tumor sites in the tissue that are indistinguishable to the naked eye. It would help physicians determine how well a drug is working in different organs of the body. Analysis of different regions in a tissue sample would facilitate evaluation of the mechanism of its drug action and its effectiveness.

Detection of Circulating Nucleosomes in Serum of Cancer Patients

In the nucleus of eukaryotic cells, DNA is associated with several protein components and forms complexes known as nucleosomes. During cell death, particularly during apoptosis, endonucleases are activated that cleave the chromatin into multiple oligo- and mononucleosomes. Subsequently, these nucleosomes are packed into apoptotic bodies and are engulfed by macrophages or neighboring cells. In cases of high rates of cellular turnover and cell death, they also are released into the circulation and can be detected in serum or plasma by Cell Death Detection-ELISAplus from Roche Diagnostics. As enhanced cell death occurs under various pathologic conditions, elevated amounts of circulating nucleosomes are not specific for any benign or malignant disorder. However, the course of change in the nucleosomal levels in circulation of patients with malignant tumors during chemotherapy or radiotherapy is associated with the clinical outcome and can be useful for the therapeutic monitoring and the prediction of the therapeutic efficacy.

Detection of Tumor Biomarkers with ProteinChip Technology

The ProteinChip Biomarker System was developed for the Expression Difference Mapping™ of several hundreds of samples per day in a single uncomplicated platform with software support for the construction of multi-marker predictive models. SELDI-based ProteinChip technology has been used for the detection of tumor biomarkers. The multimarker system has been shown to be superior over the single marker strategy and is faster. This system has been used for the detection of biomarkers of cancers of several organs including the prostate, overy, breast and lungs. HealthDigit, a protein chip system for simultaneous detection 12 cancer biomarker (C-12) in the blood, is widely used in major hospitals across China to screen for various cancers.

SELDI-TOF-MS of platelet extracts for proteomic profiling shows increased amounts of angiogenic regulatory proteins such as VEGF and endostatin in platelet but not in plasma. This is a selective sequestration process and not a simple association with the platelet surface. This novel property of platelets detects human cancers of a microscopic size undetectable by any presently available diagnostic method. This more inclusive than a single biomarker because it can detect a wide range of tumor types and tumor sizes. Relative changes in the platelet angiogenic profile permit the tracking of a tumor throughout its development, beginning from an early in situ cancer.

Glycoprotein Biomarkers of Cancer

Changes in N-linked glycosylation are known to occur during the development of cancer. For example, increased branching of oligosaccharides has been associated with metastasis and has been correlated to tumor progression in human cancers of the breast, colon and melanomas. Increases in core fucosylation have also been associated with the development of hepatocellular carcinoma (HCC). Chronic infection with the HBV is associated with >55% of all cases of HCC. Increased levels of core fucosylation, observed via glycan analysis of total serum, are associated with the development of HCC. Serum glycoproteins derived from persons diagnosed with HBV-induced HCC have a dramatically higher level of fucosylation. This change occurs on both immunoglobulin molecules and on other serum glycoproteins. Targeted glycoproteomic analysis is used to identify glycoproteins that are hyperfucosylated, and are potential biomarkers of cancer.

Gels prepared by biomolecular imprinting using lectin and antibody molecules as ligands can also be used for recognition for tumor-specific biomarker glycoproteins. The glycoprotein-imprinted gels, prepared with minute amounts of cross-linkers, can dynamically recognize tumor-specific biomarker glycoproteins by lectin and antibody ligands and induce volume changes according to the glycoprotein concentration. The glycoprotein-imprinted gels shrink in response to a target glycoprotein but nonimprinted gels expand. The glycoprotein-responsive shrinking of the imprinted gel is caused by formation of lectin-glycoprotein-antibody complexes that act as reversible cross-linking points. Glycoprotein-imprinted gels only

shrink when both lectin and antibody in the gels simultaneously recognize the saccharide and peptide chains of the target glycoprotein. As shrinking behavior of biomolecularly imprinted gels in response to glycoproteins enables the accurate detection and recognition of tumor-specific biomarker glycoproteins with potential applications for molecular diagnosis of cancer.

HER-2/neu Oncoprotein as Biomarkers for Cancer

HER-2/neu oncoprotein has been widely studied for many years and has been shown to play a pivotal role in the development and progression of breast cancer. HER-2/neu has been shown to be an indicator of poor prognosis with patients exhibiting aggressive disease, decreased overall survival and a higher probability of recurrence of disease. As evidenced by numerous published studies, elevated levels of HER-2/neu (also referred to as overexpression) are found in about 30% of women with breast cancer. Determination of a patient's HER-2/neu status may be valuable in identifying whether that patient has a more aggressive disease and would, thus, derive substantial benefit from more intensive or alternative therapy regimens. Elevated levels of HER-2/neu are found not only in breast cancer but also in several other tumor types including prostate, lung, pancreatic, colon and ovarian cancers.

Some studies suggest that in certain breast cancer patients, persistently rising HER-2/neu values may be associated with aggressive cancer and poor response to therapy, while decreasing HER-2/neu levels may be indicative of effective therapy.

Traditional HER-2/neu testing is generally limited to tissue from primary breast cancer and does not provide information regarding the HER-2/neu status in women with recurrent, metastatic breast cancer. The introduction of microtiter plate ELISA HER-2/neu testing using a serum sample now offers a less invasive diagnostic tool and provides a current assessment of a woman's HER-2/neu status over the course of disease.

IHC analysis of HER2/neu in breast carcinoma is a useful predictor of response to therapy with trastuzumab when strongly positive. Negative immunostaining is highly concordant with a lack of gene amplification by FISH. Most weakly positive overexpressors are false-positives on testing with FISH. Thus, screening of breast carcinomas with IHC and confirmation of weakly positive IHC results by FISH is an effective strategy for testing HER2/neu as a predictor of response to targeted therapy.

Humoral Proteomics

The repertoires of serum autoantibodies differ between healthy persons and cancer patients. In healthy individuals these autoantibodies are directed against a limited number of self-proteins, but in cancer patients the antibody repertoires are much further expanded with a wider range of reactivities against other proteins. Although cancer patients clearly mount humoral immune responses, they are not very effective in preventing the progression of the disease. The presence of these

new and abnormal antibody specificities indicates their potential as novel tools for early detection of cancer before clinical manifestations. Proteomics technologies, with their unique ability to identify both tumor antigens and their cognate serum autoantibodies, hold great promise in facilitating the development of early detection kits and possibly also as conduits for the isolation of tumor antigens for immunotherapy (Shoshan and Admon 2007).

Laser Capture Microdissection

Introduction of laser capture microdissection (LCM) has greatly improved the specificity of 2D PAGE for biomarker discovery, as it provides a means of rapidly procuring pure cell populations from the surrounding heterogeneous tissue and also markedly enriches the proteomes of interest. Proteomic patterns in breast cancer have been assessed by using MALDI MS and LCM (Sanders et al. 2008). Protein and peptide expression in invasive mammary carcinoma versus normal mammary epithelium and ER-positive versus ER-negative tumors were compared. Biomarker candidates were identified by statistical analysis and classifiers were developed and validated. Several of the features used in the classifiers were identified by LC-MS/MS and two were confirmed by IHC.

Membrane-Type Serine Protease-1

The cell surface protease membrane-type serine protease-1 (MT-SP1, matriptase is often upregulated in epithelial cancers. A study has shown that MT-SP1 is active on cancer cells and that its activity may be targeted in vivo for tumor detection (Darragh et al. 2010). A proteolytic activity assay with several MT-SP1-positive human cancer cell lines showed that MT-SP1 antibodies that inhibit recombinant enzyme activity in vitro also bind and inhibit the full-length enzyme expressed on cells. In contrast, in the same assay, MT-SP1-negative cancer cell lines were inactive. Fluorescence microscopy confirmed the cell surface localization of labeled antibodies bound to MT-SP1-positive cells. To evaluate in vivo targeting capability, fluorescently labeled antibodies were administered to mice bearing tumors that were positive or negative for MT-SP1. Antibodies localized to MT-SP1-positive tumors enabling visualization of MT-SP1 activity, whereas MT-SP1-negative tumors were not visualized. These findings define MT-SP1 activity as a useful biomarker for epithelial cancers using a noninvasive antibody-based method.

Proteomic Analysis of Cancer Cell Mitochondria

A combination of reverse-phase protein microarray with radiolabeled glucose metabolic studies has shown that there is a specific association between altered cytochrome c oxidase subunit levels and altered metabolism in cancer cells.

Mutations in mitochondrial DNA have been frequently reported in cancer cells. Significance of gene expression patterns is not established yet. Role of proteomics in the study of mitochondrial proteome in cancer is:

- Identification of abnormally expressed mitochondrial proteins in cancer cells is possible by mitochondrial functional proteomics.
- Proteomics can identify new biomarkers for early detection and risk assessment, as well as targets for therapeutic intervention.
- Advances in proteomics have enabled high-throughput profiling of data generated from bladder cancer-related proteins with high sensitivity and specificity, providing a wealth of information for biomarker discovery and validation (Zhang et al. 2017).

Proteomic Technologies for Detection of Autoimmune Biomarkers

There is considerable evidence for an immune response to cancer in humans, as demonstrated in part by the identification of autoantibodies against a number of tumor-associated antigens in sera from patients with different cancer types (Desmetz et al. 2008). Thus, identification of tumor-associated antigens and their associated antibodies is a promising strategy to find relevant biomarkers. Proteomic approaches such as SEREX and SERPA have enabled identification of a large numbers of antigens and their cognate autoantibodies. They have many advantages for identification of relevant autoantigens in different types of cancer. However, they are also time consuming, and lack sensibility and specificity. To circumvent these drawbacks, new proteomic techniques, based on protein or antibody arrays, allow high throughput analysis of multiple targets in a single experiment. Specific combinations of biomarkers should be identified, as they are more effective for detection of cancer compared to a single biomarker. These approaches are promising for discovery of cancer biomarkers, but also need to be further validated for clinical application on large populations.

In cancer cells, cyclic AMP-dependent protein kinase (PKA) is secreted into the conditioned medium. This PKA, designated as extracellular protein kinase A (ECPKA), is markedly up-regulated in the sera of patients with cancer and PKA inhibitors have been tested in clinical trials as a novel cancer therapy. In serum specimens, the presence of autoantibody directed against ECPKA is highly correlated with cancer. High anti-ECPKA autoantibody titers are found in the sera of patients with various cancers, whereas control groups have low or negative titers. The autoantibody method distinguishes between patients with cancer and controls better than the antigen method. In one study, the mean ECPKA activity in the sera of cancer patients was 10.98 units/mL, i.e., 5-fold higher than that of the healthy controls, and logistic analysis revealed that cancer was the only factor contributing to the elevation of ECPKA activity (Wang et al. 2007). Thus, ECPKA is a biomarker for various human cancers and can be used in cancer detection and for monitoring response to therapy along with other screening or diagnostic techniques.

SELDI-TOF MS

Surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) MS is an important tool for the rapid identification of cancer-specific biomarkers and proteomic patterns in the proteomes of both tissues and body fluids. It is useful in high-throughput proteomic fingerprinting of cell lysates and body fluids that uses on-chip protein fractionation coupled to time-of-flight separation. Within minutes, sub-proteomes of a complex milieu such as serum can be visualized as a proteomic fingerprint or 'barcode'. SELDI technology has significant advantages over other proteomic technologies in that the amounts of input material required for analysis are miniscule compared with more traditional 2D-PAGE approaches. A number of studies have used SELDI technology to identify single disease-related biomarkers for several types of cancer. For example, a modified, quantitative SELDI approach has been used to show that the levels of serum prostate-specific membrane antigen are significantly higher in patients with prostate cancer than in those with benign disease.

Serum Proteome Analysis for Early Detection of Cancer

Proteome analysis has been used for the identification of biomarkers or biomarker patterns that may allow for the early diagnosis of cancer. This tool is of special interest, since it allows for the identification of tumor-derived secretory products in serum or other body fluids. In addition, it may be used to detect reduced levels or loss of proteins in the serum of cancer patients that are present in noncancer individuals. These changes in the serum proteome may result from cancer-specific metabolic or immunological alterations, which are, at least partly, independent of tumor size or mass, thereby fascilitating early discovery.

Synthetic Biomarker-Based POC Diagnostic for Cancer

There is a need for low-cost, noninvasive methods to diagnose and treat cancer, especially in resource-limited developing countries. Molecular biomarkers combined with low-cost POC assays are a potential solution for diagnosing cancer. Synthetic biomarker technology can amplify signals from tumor proteins that would be hard to detect on their own. These proteins, matrix metalloproteinases, help cancer cells escape their original locations by cutting through proteins of the extracellular matrix, which normally holds cells in place. These synthetic biomarkers are composed of nanoparticles conjugated to ligand-encoded reporters via protease-sensitive peptide substrates (Warren et al. 2014). Upon delivery, the nanoparticles passively target solid tumors where up-regulated proteases cleave the peptide substrates and release reporters that are excreted into urine. The reporters are engineered for detection by sandwich immunoassays, and can be quantified directly from unmodified urine; furthermore, capture antibody specificity enable the probes to be multiplexed in vivo and quantified simultaneously by ELISA or paper lateral

flow assay. Synthetic biomarkers, specific for colorectal cancer, were designed and urinary detection of these was demonstrated in mouse models by paper diagnostic, which works much like a pregnancy test. This approach can be applied universally without expensive equipment or trained personnel.

Triple-Quadrupole MS for Detection of Mutant Proteins

Gene products resulting from somatic mutations are meaningful protein biomarkers, because they are not merely associated with tumors but are responsible for carcinogenesis. Altered protein products resulting from somatic mutations can be identified directly and quantified by MS. Peptides expressed from normal and mutant alleles have been detected by selected reaction monitoring of their product ions using a triple-quadrupole MS (Wang et al. 2011). As an example of this approach, the authors demonstrated that it is possible to quantify the number and fraction of mutant Ras protein present in cancer cell lines. There were an average of 1.3 million molecules of Ras protein per cell, and the ratio of mutant to normal Ras proteins ranged from 0.49 to 5.6. Similarly, they found that mutant Ras proteins could be detected and quantified in clinical specimens such as colorectal and pancreatic tumor tissues as well as in premalignant pancreatic cyst fluids. In addition to answering basic questions about the relative levels of genetically abnormal proteins in tumors, this approach could prove useful for diagnostic applications.

Targeted MS for Validation of Cancer Biomarkers in Plasma

A data-dependent triage process was used to prioritize a subset of putative plasma biomarkers from >1000 candidates previously identified using a mouse model of breast cancer (Whiteaker et al. 2011). The team used a comparable targeted mass spectrometry (MS) that relied on “accurate inclusion mass screening” and another targeted method known as selected reaction monitoring-MS, to sift through candidate biomarkers. Thirty-six proteins were verified as being elevated in the plasma of tumor-bearing animals. The final candidate biomarkers were highly credentialed so they had a high probability of being real biomarkers. The analytical performance of this pipeline suggests that it should support the use of an analogous approach with human samples. Although the biological variation among humans will undoubtedly be greater than that among mice, the pre-analytic and analytic variations associated with the technologies are the same regardless of which species is used.

Tissue Proteomics for Discovery of Cancer Biomarkers

The molecular complexity of tissue and the in vivo inaccessibility of cells within solid tumors hinder efforts to discover new diagnostic biomarkers useful for noninvasive tumor-specific molecular imaging. Use of sub-cellular fractionation of tissue,

subtractive proteomic analysis, bioinformatics and expression profiling, has identified several biomarkers induced in solid tumors at the tissue-blood interface that are accessible to agents injected intravenously. Molecular imaging validates immunotargeting and penetration of single organs and solid tumors. These biomarkers are expressed on vascular endothelium and its specialized transport vesicles (caveolae) in multiple human and rodent tumors including primary and metastatic lesions found in the liver, brain, breast, kidney, lung, intestine and prostate. Mapping tissue- and disease-modulated endothelial cell surface and caveolar proteins reveals promising biomarkers for imaging and therapy of solid tumors.

VeraTag System for Cancer Biomarkers

VeraTag® system (Monogram Biosciences/LabCorp) for profiling of pathways and cancer biomarker development involves high throughput study of tens to hundreds of genes, proteins and cell-based antigens across thousands of samples. The system makes it possible for researchers to adopt a systems biology approach towards studies of gene expression, protein expression and for applications such as cell signaling and pathway activation, protein-protein interaction and cell receptor binding. Specific molecular binding events result in the release of electrophoretically distinct reporters, which provide precise, sensitive quantitation of multiple analytes directly from cell lysates. VeraTag technology accurately quantifies the following cancer biomarkers: EGFR/HER1, HER2 and p95HER2, cMET, HGF and cMET-HGF complex, heterodimers for HER1/HER2 and HER2/HER3. HERmark® is a validated breast cancer assay for anti-HER2 therapy.

Metabolomic Biomarkers of Cancer

Compared to the cancer genome and the cancer proteome, there are few studies on the cancer metabolome. The importance of metabolomics becomes obvious if one looks at the pathways involved in cancer. Tumor cells respond to growth signals by the activation of protein kinases, altered gene expression and changes in cellular function. The transformed cells show unique anabolic characteristics, which include increased and preferential utilization of glucose through the non-oxidative steps of the pentose cycle for nucleic acid synthesis but limited de novo fatty acid synthesis and tricarboxylic acid (TCA) cycle glucose oxidation. This primarily non-oxidative anabolic profile reflects an undifferentiated highly proliferative cell phenotype and serves as a reliable metabolic biomarker to determine cell proliferation rate and the level of cell transformation/differentiation in response to drug treatment. Novel drugs effective in particular cancers exert their anticancer effects by inducing significant reversions of a few specific non-oxidative anabolic pathways. Cell transformation of various mechanisms is sustained by a unique disproportional substrate distribution between the two branches of

the pentose cycle for nucleic acid synthesis, glycolysis and the TCA cycle for fatty acid synthesis and glucose oxidation. This can be demonstrated by the broad labeling and unique specificity of [1,2-(13)C(2)]glucose to trace a large number of metabolites in the metabolome as biomarkers. Stable isotope-based dynamic metabolic profiles serve the drug discovery process by providing a powerful new tool that integrates the metabolome into a functional genomics approach to developing new drugs. It can be used in screening kinases and their metabolic targets, which can therefore be more efficiently characterized, speeding up and improving drug testing, approval and labeling processes by saving trial and error type study costs in drug testing.

Magnetic Resonance for Detecting Metabolomics Biomarkers of Cancer

When using MR metabolomics as a clinical tool in oncology, it is essential to differentiate between metabolic profiles of cancer and normal tissue. High-resolution magic angle spinning (HR-MAS), in combination with multivariate analysis, clearly discriminates the metabolic profiles of cancer tissue and normal and/or adjacent tissue in different cancer types such as breast, colorectal, prostate, and cervical cancer (Bathen et al. 2010). The most prominent differences are found in metabolites such as the choline-containing compounds (ChoCC), glycine and glucose. Multivariate analysis combines information from all metabolites detected in the tissue samples simultaneously. By interpreting these results, it is possible to identify single metabolites or ratios of metabolites that are the most differently expressed. In this way, possible biomarkers can be detected and information about specific pathways obtained

Choline Phospholipid Biomarkers of Cancer

Choline phospholipid metabolism is altered in several cancers. HR-MAS is useful but MRI and PET can also be used for choline metabolism-based diagnosis of cancer. The choline metabolite profile of tumors is characterized by elevation of phosphocholine and phosphocholine-containing compounds. They can be used as endogenous biomarkers of cancer or for monitoring the response of cancer to treatment. The enzymes directly causing this elevation such as choline kinase and phospholipases C as well as D may provide targets for anticancer drugs. Signal transduction pathways that are activated in cancer such as those mediated by receptor tyrosine kinases and EGFR also provide targets for drugs. Decrease in phosphocholine, total choline, and phosphomonoesters are potential noninvasive pharmacodynamic biomarkers for determining tumor response following treatment with choline kinase inhibitors. Choline kinase-α (ChoKα) activity is associated with drug resistant, metastatic, and malignant phenotypes, and represents a robust biomarker and therapeutic target in cancer (Arlauckas et al. 2016). Effective ChoKα inhibitors have entered clinical trials.

Hypoxia-Inducible Factor-1

Tumor hypoxia is well recognized in oncology to be a key factor resulting in treatment resistance and poor prognosis. Hypoxia leads to the expression of a number of gene products that are involved in tumor progression, invasion and metastasis formation. The most important of these proteins is thought to be hypoxia-inducible factor-1 (HIF-1), which appears to be a master regulator of the cellular response to hypoxia. HIF-1 pathway, which enables cells to sense and respond to hypoxia, is overexpressed in many cancers. HIF-1 is a transcription factor that upregulates numerous genes involved in processes such as glucose metabolism, glycolysis. pH regulation, cellular proliferation, matrix metabolism and regulation of blood vessels. HIF-1 is being explored as an attractive target for drug discovery.

HIF-1 expression is associated with a poor prognosis and treatment response in a number of tumor sites. There is some evidence that the HIF-1 pathway might be involved in gastric carcinogenesis. Immunohistochemical expression of HIF-1 target genes is associated with a poor prognosis of gastric cancer. Targeted inhibition of HIF-1 has been shown to inhibit the growth of gastric tumors in animals. Increased understanding of the importance of hypoxia and the HIF-1 pathways may, therefore, hold the key to prevention strategies, improved selection of patients for adjuvant therapy and new treatments for the disease.

Expression of endogenous biomarkers of hypoxia for the HIF-1/HIF-2 pathway is strongly associated with radiotherapy failure. Using immunohistochemical methods it is possible to identify subgroups of head and neck squamous cell carcinoma patients who are highly curable with radiotherapy, or who are excellent candidates for clinical trials on hypoxia-targeting drugs in two distinct pathways.

Detection of Drug Resistance in Cancer by Metabolic Profiling

Acquired resistance to imatinib mesylate is an increasing and continued challenge in the treatment of BCR-ABL tyrosine kinase positive leukemias as well as gastrointestinal stromal tumors. Stable isotope-based dynamic metabolic profiling (SIDMAP) studies conducted in parallel with the development and clinical testing of imatinib revealed that this targeted drug is most effective in controlling glucose transport, direct glucose oxidation for RNA ribose synthesis in the pentose cycle, as well as de novo long-chain fatty acid synthesis. Thus imatinib deprives transformed cells of the key substrate of macromolecule synthesis, malignant cell proliferation, and growth. Tracer-based MRS studies have revealed a restitution of mitochondrial glucose metabolism and an increased energy state by reversing the Warburg effect, consistent with a subsequent decrease in anaerobic glycolysis. In vitro SIDMAP studies, which involved myeloid cells isolated from patients who developed resistance against imatinib, have indicated that non-oxidative ribose synthesis from glucose and decreased mitochondrial glucose oxidation are reliable metabolic signatures of drug resistance and disease progression. There is also evidence that imatinib-resistant cells utilize alternate substrates for macromolecule synthesis to

overcome limited glucose transport controlled by imatinib. The main clinical implications involve early detection of imatinib resistance and the identification of new metabolic enzyme targets with the potential of overcoming drug resistance downstream of the various genetic and BCR-ABL-expression derived mechanisms. Metabolic profiling is an essential tool used to predict, clinically detect, and treat targeted drug resistance. This need arises from the fact that targeted drugs are narrowly conceived against genes and proteins but the metabolic network is inherently complex and flexible to activate alternative macromolecule synthesis pathways that targeted drugs fail to control.

Plasma Free Amino Acids Profiling in Cancer

Profiling of plasma free amino acids (PFAAs) is a promising approach for early detection of cancer. Focused metabolomic approach with PFAAs overcomes some of the problems associated with metabolomic profiling of cancer that include include the need for measuring a huge number of metabolites, data-redundancy problems, and high cost. In a study using large number of plasma samples from cancer patients, PFAA levels were measured using HPLC, and ESI-MS (Miyagi et al. 2011). Univariate analysis revealed significant differences in the PFAA profiles between the controls and the patients with any of the 5 types of cancer – lung, gastric, colorectal, breast, or prostate cancer – even in patients with asymptomatic early-stage disease. These findings indicate that PFAA profiling from a single blood sample has considerable potential for improving cancer screening and diagnosis and understanding disease pathogenesis.

Urinary Metabolomic Biomarkers of Cancer

Metabolomics techniques have been to identify metabolites in urine of patients with kidney cancer, but their levels differ from the same metabolites in nonkidney cancer patients (Kim et al. 2011). These authors found that quinolinate, 4-hydroxybenzoate, and gentisate are differentially expressed at a false discovery rate of 0.26, and these metabolites are involved in common pathways of specific amino acid and energy metabolism, consistent with high tumor protein breakdown and utilization. When added to 4 different (3 kidney cancer-derived and one “normal”) cell lines, several of the significantly altered metabolites, quinolinate, α-ketoglutarate, and gentisate, showed increased or unchanged cell proliferation that was cell line-dependent. Further evaluation as well as validation of the specific potential biomarkers using a larger sample size, will be required before use of these biomarkers for diagnosis and therapy of kidney cancer.

Identification of volatile organic metabolites (VOMs) as biomarkers that can accurately diagnose the onset of cancer using non-invasively collected urine specimens is ideal for early detection. This approach has been applied to diagnosis of vaious cancers. A study of the urinary metabolomic profile of breast cancer patients

and healthy individuals as controls has explored VOMs as potential biomarkers in breast cancer diagnosis at early stage (Silva et al. 2012). Solid-phase microextraction (SPME) using CAR/PDMS sorbent combined with gas GC-MS was applied to obtain metabolomic patterns. Ketones and sulfur compounds were the chemical classes with highest contribution for both groups. Results showed that excretion values of 6 VOMs among the total of 79 detected were found to be statistically different. A significant increase in the peak area of (-)-4-carene, 3-heptanone, 1,2,4-trimethylbenzene, 2-methoxythiophene and phenol, in VOMs of cancer patients relatively to controls was observed. Statistically significant lower abundances of dimethyl disulfide were found in cancer patients. Another study found that urinary biomarkers disturbed in several metabolic pathways epithelial ovarian cancer patients, included those associated with nucleotide metabolism, histidine metabolism, tryptophan metabolism, and mucin metabolism (Zhang et al. 2013).

Epitomics for the Early Detection of Cancer

The word "epitomics" derives from the combined words of epitope and omics. An epitope is a functional recognition site that binds by a specific MAb. Epitomics studies all epitopes of the proteome in an organism. The understanding of epitope reveals the functions of these proteins. Efforts toward the development of early detection assays for cancer have traditionally depended on single biomarker molecules. Current technologies have been disappointing and have not resulted in diagnostic tests suitable for clinical practice. In an innovative approach, discovery of biomarker panels is directed in an unbiased fashion by cloning a large panel of epitopes or tumor antigens, rather than individual biomarkers without a previous notion of their function. The binding properties of serum antitumor antibodies on microarrays and advanced bioinformatics tools have led to a panel of diagnostic antigens. The sequences identified by this technology can lead to the discovery of novel disease-related proteins that have diagnostic value for the presymptomatic detection of cancer. This approach can detect these autoantibodies in the sera of Stage I ovarian cancer patients. There are numerous advantages of employing serum antibodies as the analytes, not the least of which is the ability to rapidly adapt these assays to standard clinical platforms.

Epigenetic Biomarkers of Cancer

Profound changes in the epigenetic landscape of cancer cells underlie the development of human malignancies. These changes include large-scale DNA methylation changes throughout the genome as well as alterations in the compendium of post-translational chromatin modifications. Epigenetic aberrations impact multiple steps during carcinogenesis, ultimately promoting the selection of neoplastic cells with

increasing pathogenicity. Identification of these alterations for use as predictive and prognostic biomarkers has been a highly sought after goal. Recent advances in the field have not only greatly expanded our knowledge of the epigenetic changes driving neoplasia but also demonstrated their significant clinical utility as cancer biomarkers. These biomarkers have proved to be useful for identifying patients whose malignancies are sensitive to specific cytotoxic chemotherapies and may hold promise for predicting which patients will benefit from newer targeted agents directed at oncogenes (Chan and Baylin 2012).

Detection of Biomarkers of DNA Methylation

Methylated DNA (meDNA) is a very stable carrier of epigenetic information that is directly involved in tumor formation and progression. Genes that are often methylated in tumors are termed tumor biomarkers because their methylation can be used to detect the disease. Utilization of methylated DNA markers is superior to reliance on other types of markers for numerous reasons, including:

- Methylation is directly responsible for regulation of many cancer genes.
- DNA is chemically and biologically more stable than RNA and many other types of biomarkers
- The levels of gene methylation in cancer cells is very constant and not subject to fluctuations as seen for expressed products such as RNA, proteins, and metabolites.
- meDNA assays are inherently very sensitive (MethylPlex can detect as little as 1 cancer cell in a mixture with 10,000 normal cells).
- meDNA is a universal analyte for diagnostics, because the same technology, instrumentation, and data analysis methods can be used to detect many types of cancer and other diseases.

Some problems with DNA methylation analysis are:

- Probability of methylation for each gene is less than 100%.
- Clinical samples are heterogeneous.
- Quantitative assay of methylation is difficult.

Because of variations of methylation of genes in different types of cancer and various stages of the disease, the methylation of any single gene may not provide sufficient information. However, a test based on the pattern of methylation in a number of genes would have the high sensitivity and specificity required for an accurate diagnosis and prognosis. Use of a systematic biological screen has enabled identification of multiple genes that are methylated with high penetrance in primary lung, breast, colon, and prostate cancers. The cross-tumor methylation pattern observed for these novel biomarkers suggests that a partial promoter hypermethylation signature for these common malignancies has been identified. These data suggest that while tumors in different tissues vary substantially with respect to gene

expression, there may be common features in their promoter methylation profiles that could form the basis for a new early detection screen for certain cancers.

Methyl-BEAMing technology enables absolute quantification of the number of methylated molecules in a sample (Li et al. 2009). Individual DNA fragments are amplified and analyzed either by flow cytometry or NGS and as few as one methylated molecule can be enumerated in ~5000 unmethylated molecules in DNA from plasma or fecal samples. Using methylated vimentin as a biomarker in plasma samples, methyl-BEAMing detected 59% of cancers. In addition to diagnostic and prognostic applications, this digital quantification of rare methylation events is applicable to preclinical assessment of new epigenetic biomarkers and quantitative analyses in epigenetic research.

In early-stage CRC, sensitivity of detection by methyl-BEAMing technology is four times more than that obtained by assaying CEA (Li et al. 2009). With stool samples, methyl-BEAMing detected 41% of cancers and 45% of advanced adenomas.

Changes in gene expression due to epigenetic regulation can be reversed by chemicals, and this approach opens up a novel approach in cancer treatment in addition to diagnosis. Methylation in noncancerous tissues is now attracting attention as a biomarker of risk of cancer, and is emerging as a target for cancer prevention. Many of the tests are in development commercially and will be described briefly.

Epigenomics Marker Machine for DNA Methylation Biomarkers

Epigenomics AG has developed a proprietary technology consisting of a combination of chemically treated DNA, highly multiplexed amplifications, high-density arrays, and MALDI-MS. This technology makes the detection of hundreds of thousands of DNA methylation signals a reality. These signals can be digitized into a long string of ones and zeros, creating a Digital Phenotype that reflects genetic activity in a particular cell or tissue, i.e. whether it is functioning normally or whether it is sick.

A large-scale genome wide screening effort of all major human tumors for DNA methylation biomarkers in tissue and serum has led to the discovery of >200 such biomarkers. Methylation-based DNA biomarkers will allow the detection of disease much earlier than currently available diagnostics. This will allow physicians to develop the best treatment for existing disease, monitor the effects of treatment and identify people at risk of developing disease. Such novel DNA methylation biomarkers could form the backbone of future molecular diagnostics. Another fascinating finding is that dozens of these biomarkers are derived from genes not yet implicated in cancer development, making them highly interesting candidates as pharmaceutical targets. It is generally accepted that DNA methylation is involved in global transcriptional regulation of the human genome. Hypermethylation can lead to decreased gene activity, for instance blocking genes that protect against cancer. On the flip side, hypomethylation of normally inactive genes often leads to their activation and acceleration of the disease process. Validated panels of informative

DNA methylation positions for various types of cancer diagnostics such as differential diagnosis, treatment planning tests, monitoring tests, as well as serum based early screening tests are expected to be developed. The convenience, performance and cost-effectiveness of these tests will make them ideal for mass screening programs for all major cancer types.

Histone Deacetylase

Levels of histone acetylation are tightly modulated in normal cells, and alterations of their regulating mechanisms have been shown to be involved in carcinogenesis. Flow cytometry can successfully detect modifications induced by by the histone deacetylase (HDAC) inhibitor treatment in vivo. Multiparameter analysis of histone acetylation and expression of molecular biomarkers, DNA ploidy, and/or cell cycle kinetics can provide a quick and statistically reliable tool for the diagnosis and evaluation of treatment efficacy in clinical trials using HDAC inhibitors. HDAC inhibitors induce differentiation of breast cancer cells and inhibit tumor growth. HDAC-1 expression correlates with steroid hormone receptor-, Her2/neu- and proliferation status of tumors as well as to overall as well as cancer free survival. Multivariate analysis shows that HDAC-1 is an independent prognostic biomarker and evaluation of HDAC-1 protein expression enables a more precise assessment of the prognosis of breast cancer patients. HDAC-1 expression analysis might be clinically useful to facilitate an individual, risk-directed, adjuvant systemic therapy in breast cancer patients.

MDScan™ Microarray Technology

MDScan™ (GenomicTree) technology for the systematic and comprehensive genome-wide discovery of epigenetically silenced genes uses affinity-based methyl DNA enrichment, a bisulfite-free method, for selective enrichment of methylated DNA. The selectively isolated methyl DNA can be used for microarray analysis. This technology does not depend on bisulfite modification, restriction endonucleases or specific antibodies and will lead to the discovery of novel methylation biomarkers for early detection of cancer, determination of tumor stage, risk of recurrence, and prediction of response to drug therapy.

Mucins as Epigenetic Biomarkers in Epithelial Cancers

Genes encoding mucins have been shown to be regulated by DNA methylation and histone modifications in epithelial cancer cells. These genes encode either secreted glycoproteins necessary for epithelial homeostasis or membrane-bound glycoproteins that participate in tumor progression. The important biological functions played by these large molecules in pathophysiology of the epithelia make them key

genes to target for new therapeutic strategies and new diagnostic and/or prognostic tools in cancer. Mucin genes may be regulated by miRNAs and also regulate miRNA activity. Epigenetic regulation of mucin genes has a great potential to provide new diagnostic as well as prognostic biomarkers, help tumor classification and form the basis of new therapeutic targets for the clinician and the pathologist (Van Seuningen and Vincent 2009).

PCR with Bisulfite for Detecting DNA Methylation Biomarkers in Cancer

Several traditional and new PCR-based methods have been developed for detecting DNA methylation at single loci. All have characteristic advantages and disadvantages, particularly with regard to use in clinical settings. In order to detect methylation patterns on DNA, one needs greater amounts of it than can be extracted from a small patient sample. In order to achieve this, one can increase the amount of DNA extracted from minute samples rather than use larger samples. Use of PCR for amplifying DNA results in the loss all information on the positions of methylcytosine.

To overcome the limitations of conventional PCR, Epigenomics Inc. uses a procedure that is based on modification of all non-methylated cytosines to a different base, uracil, by the chemical bisulphite. Uracil's hybridization behavior is identical to that of thymine. Thus, in DNA treated with bisulphite, methylcytosine can easily be detected by hybridizing to guanine. This enables the use of variations of established methods of molecular biology, most notably PCR, hybridization, oligonucleotide-arrays, and mass-spectrometry. The remaining cytosines are present in the sequence context 5′-cytosine-guanine-3′ (CpG). The total number of CpG positions in the human genome is ~40 million, up to 10% of which are located in non-repetitive, relevant sections of DNA.

MDxHealth Inc's methylation-specific PCR (MSP) can rapidly assess the methylation status of virtually any group of CpG sites within a CpG island, independent of the use of methylation-sensitive restriction enzymes. An MSP assay entails initial modification of DNA by sodium bisulfite, converting all unmethylated, but not methylated, cytosines to uracil, and subsequent amplification with primers specific for methylated versus unmethylated DNA. MSP requires only small quantities of DNA, is sensitive to 0.1% methylated alleles of a given CpG island locus, and can be performed on DNA extracted from paraffin-embedded samples. MSP eliminates the false positive results inherent to previous PCR-based approaches, which relies on differential restriction enzyme cleavage to distinguish methylated from unmethylated DNA. Patent-protected MSP platform is the only scalable technology that enables a sensitive and specific detection of methylated genes in a background of normal cells, critical for early diagnosis or detection of micrometastases in serum, saliva or sputum samples. The process employs an initial bisulfite reaction to modify the DNA, followed by PCR amplification with specific primers designed to distinguish methylated from unmethylated DNA. This specific alteration enables the detection of a few cancer cells embedded in otherwise normal tissue. This process is universal and can be applied to the detection of promoter hypermethylation of relevant tumor suppressor genes or any other cellular genes related to cancer. A practical

aspect of this diagnostic marker strategy is the concentration on specific genes known to play an important role in tumorigenesis. This approach is far less labor intensive and more amenable to high throughput screening than microarray assays.

Orion's MethylScreen® technology leverages biomarkers discovered using its MethylScope technology to develop of a new class of oncology diagnostic kits. MethylScreen is a reliable enzyme-based real-time PCR technology that is compatible with testing platforms widely used in clinical laboratories. The sensitivity of MethylScreen assays enables Orion's scientists to measure unique qualities of epigenetic DNA that are indicative of disease progression. MethylScreen assays provide critical clinical information about disease progression using blood serum and other easily collected patient samples. MethylScreen is unique in that it is the only platform that does not rely on bisulfite conversion, a harsh chemical process that has been shown to destroy more than 94% of the tumor DNA in patient samples.

MethySYBR is a SYBR green-based PCR assay for the dual analysis of DNA methylation and CpG methylation density (Lo et al. 2009). MethySYBR begins with multiplex PCR to enable the simultaneous amplification of many discrete target alleles in a single reaction using bisulfite-converted DNA. In the second round of PCR, the specific methylated target is quantified from multiplex products using both nested methylation-independent and methylation-specific primer sets. Moreover, the use of SYBR green dye during quantitative PCR enables melting curve analysis of target amplicons to determine the methylation density of CpG sites on target alleles. To establish proof of principle, two cancer-specific methylated genes, RASSF1A and OGDHL, were assessed by MethySYBR. MethySYBR sensitively detected methylated alleles in the presence of a 100,000-fold excess of unmethylated allele. Furthermore, MethySYBR was shown to be capable of analyzing minute amounts of DNA from paraffin-embedded tissue. Therefore, the MethySYBR assay is a simple, highly specific, highly sensitive, high-throughput, and cost-effective method that is widely applicable to basic and clinical studies of DNA methylation.

PCR-based methods that use sodium bisulfite-treated DNA as a template are generally accepted as the most analytically sensitive and specific techniques for analyzing DNA methylation at single loci (Kristensen and Hansen 2009). A number of new methods, such as methylation-specific fluorescent amplicon generation (MS-FLAG), methylation-sensitive high-resolution melting (MS-HRM), and sensitive melting analysis after real-time methylation-specific PCR (SMART-MSP), now complement the traditional PCR-based methods and promise to be valuable diagnostic tools. In particular, the HRM technique shows great potential as a diagnostic tool because of its closed-tube format and cost effectiveness.

Detection of Methylated DNA in Serum and Urine

Detection of methylated DNA in serum and urine are the best approaches for developing practical non-invasive tests for cancer. The challenge is to implement a test for those genes using a noninvasive protocol. Currently, there are two significant barriers to noninvasive testing.

1. meDNA tests require a large amount of cancer DNA, which is often not present in the blood or urine of cancer patients, especially during the initial stages of disease or upon recurrence.
2. meDNA tests use a special chemistry, called bisulfite conversion, and proprietary amplification technologies that make testing complicated and expensive.

The MethylPlex technology (Rubicon Genomics) has solved both of these problems by completely avoiding bisulfite chemistry and improving technical sensitivity by a factor of 10 to 100 times, so that the methylation of many genes can be reliably detected from serum and urine.

Integrated Platform for Genetic and Epigenetic Analysis

An integrated genetic analysis platform based on MALDI-TOF MS is used to correlate SNPs with differences in allele-specific expression, and on the basis of this information to examine possible variations in DNA methylation patterns being causative of these differences. This approach has been validated using an established model based on allele-specific transcript levels of the human tumor protein 73, indicating the suitability of the MassARRAY platform (Agena Biosciences) for assaying both stable and dynamic variation at the DNA and RNA level.

Nanobiotechnology for Early Detection of Cancer to Improve Treatment

Cancer is easier to treat and less likely to develop drug resistance when treatment is started very early. Cancer cells in very early stages are less likely to have mutations that make them resistant to treatment. However, cancer cells themselves may be difficult to detect at an early stage but they leave a fingerprint, i.e., a pattern of change in biomarker proteins that circulate in the blood. There may be 20–25 biomarkers, which may require as many as 500 measurements, all of which should be made from a drop of blood obtained by pinprick. Thus nanoscale diagnostics will play an important role in this effort.

Aptasensor for Electrochemical Detection of Exosomes

Exosomes are small (50–100 nm in diameter) vesicles secreted from various mammalian cells. Exosomes have been correlated with cancer antigens as well as anticancer immune responses and may represent cancer biomarkers. An aptamer-based electrochemical biosensor has been developed for quantitative detection of

exosomes (Zhou et al. 2016). Aptamers specific to exosome transmembrane protein CD63 were immobilized onto gold electrode surfaces and incorporated into a microfluidic system. Probing strands pre-labeled with redox moieties were hybridized onto aptamer molecules anchored on the electrode surface. In the presence of exosomes these beacons released probing strands with redox reporters causing electrochemical signal to decrease. These biosensors could be used to detect as few as 1×10^6 particles/mL of exosomes, which represents 100-fold decrease in the limit of detection compared to commercial immunoassays relying on anti-CD63 antibodies. Given the importance of exosome-mediated signal transmission among cells, this study may represent an important step towards development of a simple biosensor that detects exosomes without washing or labeling steps in complex media.

Nanowire Biosensors for Detection of Cancer Biomarkers

Nanowires can electronically detect a few proteins molecules along with other biomarkers that are early signs of cancer. Nanowire sensors are in development at California Institute of Technology (Pasadena, CA) for very early diagnosis of cancer, when there are just a few thousand cells. Nanowires in a set are coated with different compounds, each of which binds to a particular biomarker, and changes the conductivity of the nanowire that can be measured. Thousands of such nanowires are combined on a single chip that enables identification of the type of cancer. Currently such a chip can detect between 20 to 30 biomarkers and is being used for the early diagnosis of brain cancer.

NP-Peptide Complexes for Detection of Cancer Biomarkers in Urine

Exogenously administered ‘synthetic biomarkers’ composed of mass-encoded peptides conjugated to nanoparticles (NPs) have been developed for noninvasive urinary monitoring to detect cancer biomarkers (Kwong et al. 2012). The NP complexes accumulate at the tumor site, where matrix metalloproteinases (MMPs) from cancer cells are expected to cleave the NP-bound peptides, releasing them into the bloodstream. The peptides would then accumulate in the kidneys and be excreted in the urine, which could be analyzed using MS. The NPs are engineered to express 10 different peptides, each with a specific corresponding MMP and size making them distinguishable by MS. In mouse models of liver cancer, these agents were shown to substantially improve early detection of cancer compared with current clinically used blood biomarkers. This method has potential for development as point-of-care diagnostics to detect metastasis and measure tumor response to chemotherapy.

Ultrasound Radiation to Enhance Release of a Tumor Biomarker

In studies on tumor-bearing mice, application of low-frequency ultrasound to tumor cells has been demonstrated to enhance the release of CEA, a biomarker of cancer, which can be measured in the blood (D'Souza et al. 2009). It was further established that this release is specific to the direct application of the ultrasound to the tumor, enabling a method for localization of biomarker production. Blood concentrations of that biomarker rose significantly only when ultrasound energy was directed to tumor sites but not when the ultrasound beam was focused on non-tumor-bearing tissues. This work will enable the detection of cancer in the pre-symptomatic stage using a relatively simple and noninvasive strategy. Future work using image-guided focused ultrasound to radiate tumors with ultrasound should help to bring together the currently separate fields of in vitro diagnostics and in vivo imaging and facilitate the development of personalized medicine. There is no significant regulatory impediments to the integration of this method into clinical practice, as ultrasound is already widely used in the clinic. It will be necessary to optimize the technique for use in humans and it will not work for all tumor types, e.g. lung or bone-marrow cancers, because ultrasound is impeded by bony structures and air-filled zones in the body. The proof of principle has been established for a single biomarker, CEA, and other biomarkers may prove more difficult to measure by this approach.

In Vivo Imaging of Cancer Biomarkers

Various 'Omic' approaches are providing comprehensive 'snapshots' of biomarkers of cancer, but imaging can take this information a step further, showing the activity of these biomarkers in vivo and how their location changes over time. Advances in experimental and clinical imaging are likely to improve the understanding of cancer at the systems level and, ultimately, should enable doctors not only to locate tumors but also to assess the activity of the biological processes within these tumors and to provide 'on the spot' treatment (Weissleder and Pittet 2008). Several technologies described earlier can be used for in vivo imaging of biomarkers in cancer. The best known of these are CT, MRI and PET.

Computer Tomography

Computer tomography (CT) is used not only for diagnosis but also measurement of volume of a tumor. 3D tumor imaging is better as a "surrogate endpoint" of measuring drug responsiveness rather than the unidimensional measurements currently used. Tumor reduction assessed by high-resolution CT has been used successfully as an endpoint in a phase II trial of pazopanib, an oral angiogenesis inhibitor

targeting VEGFR, PDGFR, and c-kit in patients with stage I-II NSCLC. CT, however, measures only the size but not metabolic and other changes in cancer at molecular level in response to treatment, that may be better indicators of response.

Optical Systems for In Vivo Molecular Imaging of Cancer

Some molecular specific contrast agents for molecular imaging are based on gold nanoparticles, which are attached to probe molecules with high affinity for specific cellular biomarkers. The application of gold bioconjugates for vital imaging of pre-cancers has been shown by using cancer cell suspensions, 3D cell cultures, and neoplastic fresh cervical biopsies. Gold conjugates as contrast agents have potential to extend the ability of vital reflectance microscopies for in vivo molecular imaging. They can potentially enable combined screening, detection, and therapy of disease using inexpensive imaging systems; such tools could allow mass screening of diseases such as cancer in resource-poor settings.

In order to use such optical systems to image molecular features of cancer, it will be necessary to deliver sufficient contrast agent to tissue so that a reasonable signal to noise ratio can be obtained. Before contrast agents can be used in human subjects, extensive animal studies must be carried out to evaluate any potential toxicity of these contrast agents and delivery formulations. In spite of these concerns, these contrast agents and imaging systems have the potential to significantly impact current clinical practice.

Positron Emission Tomography

During the past decade, positron emission tomography (PET) has been increasingly developed for imaging and quantifying molecular mechanisms in oncology. The technique uses radionuclides to label molecules, which can then be imaged in man. PET with 18F-fluorodeoxy-glucose (FDG) as a radioactive tracer for glucose metabolism is currently an effective and highly utilized tool for the diagnosis and management of cancer. Growing tumors consume glucose and show up as bright spots on PET, which can disappear a week after a patient begins chemotherapy, signaling possible remission. Waiting for demonstrable tumor shrinkage on computed tomography scans takes another six months. Seeing whether a patient actually lives longer could take years. Usually, changes in PET results are not dramatic enough to be seen by the naked eye but the signal must be interpreted through a series of calculations gauging the concentration of a radioactive tracer.

Continuing technological improvements in imaging, including higher resolution, better attenuation correction, and multimodality image registration, will further improve the efficacy of this method. The most significant improvements will come from the wide variety of tracers now being developed to image other metabolic pathways, and to identify cancer by specific biochemical, physiological, and genetic characteristics. Developments in PET mean that a wider range of molecules can

now be labeled with isotopes and old and new molecular targets for anticancer therapy can be probed, imaged and quantified in vivo in man. The inherent sensitivity and specificity of PET is unrivalled because it can image molecular interactions and pathways, providing quantitative kinetic information down to the subpicomolar level. Molecular imaging has the potential to assist in the optimization of molecular-based targeted therapies in cancer and to investigate the function of the genome.

Imaging of Tumor Oxygenation and Microvascular Permeability by MRI

Increased vascular permeability and disturbance of blood flow impairs delivery of oxygen and drugs to tumors leading to treatment resistance. Tissue oxygen concentration and microvascular permeability can be visualized simultaneously by using Overhauser enhanced MRI (a hyperpolarized 1H-MRI) and OX63, an oxygen-sensitive contrast agent (Matsumoto et al. 2009). Application of this methods for imaging murine tumors showed that tumor regions with high vascular permeability were also hypoxic with an inverse correlation between tumor vascular leakage and oxygen concentration. This imaging technique may be useful for the assessment of changes in vascular permeability and oxygenation of tumor in response to chemotherapy, radiotherapy, or antiangiogenic treatment.

Xenon-enhanced MRI

Capability of MRI for monitoring multiple cancer biomarkers simultaneously has been extended by using xenon as an imaging agent. Encapsulation of a single atom of xenon within a cage made of cryptophane provides a sensitive reporter of changes outside the cage. When the cage encounters a specific cancer protein, the xenon molecule emits a telltale signal that can be tracked by MRI. Based on this principle, new biosensors are being generated that will identify biomarkers associated with cancers of the lungs, brain and pancreas. Thus MRI can be used to detect aberrant proteins that cause cancer in humans before the actual formation of a tumor.

Kallikrein Gene Family and Cancer Biomarkers

The tissue kallikreins are serine proteases that are encoded by highly conserved multi-gene family clusters in rodents and humans. In vitro biochemical studies show that some kallikreins can auto-activate and others can activate each other, suggesting that the kallikreins may participate in an enzymatic cascade similar to that of the coagulation cascade. Human tissue kallikrein genes, located on the long arm of chromosome 19, are a subgroup of the serine protease family of proteolytic enzymes. Human kallikrein locus has now been extended and includes 15 tandemly located genes. These genes, and their protein products, share a high degree of

homology and are expressed in a wide array of tissues, mainly those that are under steroid hormone control. Kallikreins (KLK4-KLK15) have been associated with several types of cancer. For example, hK4, hK5, hK6, hK7, hK8, hK10, hK11, hK13 and hK14 are emerging biomarkers for ovarian, breast, prostate and testicular cancer. New evidence raises the possibility that some kallikreins are directly involved with cancer progression. KLKs are also considered as promising biomarkers for personalized oncology, especially for prediction and monitoring of patients' response to chemotherapy (Kontos and Scorilas 2012).

Detection of CTCs as Biomarkers of Cancer

There is need for noninvasive diagnostic method to confirm the presence of cancer. Blood samples have been analyzed for CTCs as biomarkers by using nucleic acid methods to isolate tumor-associated or tumor-specific mRNA. Detection of extremely low concentrations of rare cancer cells in the blood is still a challenge. The preferred method of detection, automated digital microscopy (ADM), is too slow to scan the large substrate areas. Fiber-optic array scanning technology (FAST) applies laser-printing techniques to the rare-cell detection problem. With FAST cytometry, laser-printing optics is used to excite 300,000 cells per sec, and emission is collected in an extremely wide field of view, enabling a 500-fold speed-up over ADM with comparable sensitivity and superior specificity. The combination of FAST enrichment and ADM imaging has the performance required for reliable detection of early-stage cancer in blood.

The CellTracks® AutoPrep® System (Immunicon Corporation) is an automated sample preparation system for immunomagnetic cell capture and fluorescence staining of rare cells. It is used with the company's reagent kits to automate and standardize the isolation of circulating tumor cells. The CellTracks® Analyzer II is a semi-automated fluorescence microscope that is used to count and characterize the immuno-magnetically selected cells based on the fluorescence signals of the cells. Immunicon's technology for the quantification and characterization of rare cells is being used in drug development trials as efficacy biomarkers, for risk stratification and to monitor expression levels of proteins associated with targeted therapy. Detection of CTCs using immunomagnetics before initiation of first-line therapy in patients with metastatic breast cancer is highly predictive of progression-free survival and overall survival. Increased numbers of circulating endothelial cells (CECs) are observed in peripheral blood of cancer patients, which may contribute to tumor growth through the process of angiogenesis. Characterization of these cells by study of gene expression profiles of immunomagnetically enriched CECs may provide biomarkers to evaluate treatment efficacy. This technology can aid in appropriate patient stratification and design of tailored treatments.

CellSearch (Veridex), based upon immunomagnetic technologies and constituted by magnetic nanoparticles coated with anti-EpCAM antibodies, currently represents one of the best systems of CTCs detection. It is approved by the FDA. Prior to

the start of a new line of chemotherapy, CTC identification through CellSearch has prognostic value in breast, prostate and colorectal cancer (O'Flaherty et al. 2012).

Although extremely rare, CTCs represent a potential alternative to invasive biopsies as a source of tumor tissue for the detection, characterization and monitoring of non-hematologic cancers. The ability to identify, isolate, propagate and molecularly characterize CTC subpopulations could further the discovery of cancer stem cell biomarkers and expand the understanding of the biology of metastasis. Current strategies for isolating CTCs are limited to complex analytic approaches that generate very low yield and purity. A unique microfluidic platform (the 'CTC-chip') is capable of efficient and selective separation of viable CTCs from peripheral whole blood samples, mediated by the interaction of target CTCs with antibody (EpCAM)-coated microposts under precisely controlled laminar flow conditions, and without requisite pre-labeling or processing of samples (Nagrath et al. 2007). The CTC-chip has successfully identified CTCs in the peripheral blood of patients with metastatic lung, prostate, pancreatic, breast and colon cancer in 99% of samples. Given the high sensitivity and specificity of the CTC-chip, its potential utility was tested in monitoring response to anticancer therapy. In a small cohort of patients with metastatic cancer undergoing systemic treatment, temporal changes in CTC numbers correlated reasonably well with the clinical course of disease as measured by standard radiographic methods. Thus, the CTC-chip provides a new and effective tool for accurate identification and measurement of CTCs in patients with cancer. It has broad implications in advancing both cancer biology research and clinical cancer management, including the detection, diagnosis and monitoring of cancer.

A massively parallel, multigene-profiling nanoplatform can compartmentalize and analyze hundreds of single CTCs (Park et al. 2016). After high-efficiency magnetic collection of CTC from blood, a single-cell nanowell array performs CTC mutation profiling using modular gene panels. This approach has enabled multigene expression profiling of individual CTCs from NSCLC patients with high sensitivity. This may facilitate highly sensitive, yet minimally invasive characterization of lung cancer for therapy prediction and monitoring. In the near future, further refinements of techniques for isolation of tumor cells in the blood and their characterization will be a powerful diagnostic tool for facilitating cytogenetics analysis analysis, early detection of cancer, localization of tumor, therapy selection, and determination of chemoresistance.

Applications of Cancer Biomarkers

The main clinical applications of cancer biomarkers are:

- Classification of tumors
- Prognosis of disease progression
- Prediction of response to therapy
- Monitoring of response to therapy

Use of Biomarkers for Cancer Classification

Cancer Classification Using Microarrays

Classification of a cancer based on gene expression profile is important for personalizing cancer therapy. In the process of expression profiling, robotically printed DNA microarrays are used to measure the expression of tens of thousands of genes at a time; this creates a molecular profile of the RNA in a tumor sample. A variety of analytic techniques are used to classify cancers on the basis of their gene-expression profiles. There are two general approaches. In an unsupervised approach, pattern-recognition algorithms are used to identify subgroups of tumors that have related gene-expression profiles. In a supervised approach, statistical methods are used to relate gene-expression data and clinical data. Determination of tumor marker genes from gene expression data requires bioinformatic tools because expression levels of many genes are not measurably affected by carcinogenic changes in the cells. These molecular biomarkers give valuable additional information for tumor diagnosis/ prognosis and will be important for the development of personalized therapy of cancer.

Gene expression microarray technology is helpful in all phases of the discovery, development and subsequent use of new cancer therapeutics, e.g., the identification of potential targets for molecular therapeutics. It can be used to identify molecular biomarkers for proof of concept studies, pharmacodynamic endpoints and prognostic markers for predicting outcome and patient selection. Expression profiling can be used alongside gene knockout or knockdown methods such as RNA interference.

Proteomic Classification of Cancer

The use of rapid, high throughput MS-based fingerprints of peptides and proteins may prove to be valuable for new molecular classification of human tumors and disease stages. Coupled with LCM, high-density protein arrays and antibody arrays will have a substantial impact on proteomic profiling of human cancers.

Use of Biomarkers for Early Detection of Cancer

Although plasma tumor biomarkers are widely used clinically for monitoring response to therapy and detecting cancer recurrence, only a limited number of them have been used effectively for the early detection of cancer. A review of the use of cancer biomarkers in the US shows that only PSA, cancer antigen 125 and α-fetoprotein have been clinically used for the early detection of prostate, ovarian and liver cancers, respectively (Meany et al. 2009). Few plasma tumor biomarkers

have been used effectively for the early detection of cancer, mainly because of their limited sensitivity and/or specificity. Several approaches are being developed to improve the clinical performance of tumor biomarkers for the early detection of cancer.

Applications of Biomarkers for Cancer Diagnosis

Methylated DNA Sequences as Cancer Biomarkers

To identify and overcome barriers in the application of methylated genes as cancer biomarkers and to promote validation studies of these biomarkers, the NCI Early Detection Research Network (EDRN) joined forces in 2005 with the National Institute of Standards and Technology (NIST) to conduct a workshop on Standards and Metrology for Cancer Detection and Diagnostics focusing upon DNA methylation. The objectives of the workshop were:

- To evaluate methods and standards for robust, sensitive, and preferentially, quantitative measurements of DNA methylation in clinical specimens.
- To evaluate demands stemming from different types of specimens (e.g. tissue versus biological fluids).
- To identify and evaluate variables (e.g. amount of DNA template) influencing the robustness of the particular assay.
- To evaluate the need, and develop recommendations, for Standard Reference Materials for the discovery and validation of methylated DNA biomarkers (including cross-validation between laboratories and platforms).
- To evaluate the need and make recommendations regarding the necessity to establish a common collection of data standards that can be used to transmit cancer-related clinical research data among investigators, clinicians, and regulators.

The results of the Workshop were published (Kagan et al. 2007). It was clear from this workshop that one standard cannot be developed for addressing all the applications for methylation in the basic and translational research fields as well as the clinical testing. The best technology depends on the question being asked. However, the development of standard assays will require standard specimens for clinical comparison. The most straightforward set of specimens are tumor cell lines, which can be regenerated and provide an unlimited source of DNA. Tumor tissue and adjacent tissue from a cancer that is common and always resected, such as colon cancer, could be a second valuable standard for assay validation. It also is clear that there is a pressing need for perhaps unexciting, but important, studies to determine the optimal parameters for choice, storage, and preparation of clinical specimen for DNA isolation, bisulfite modification, and technology controls. The conclusions of this workshop about the desirable characteristics of methylated DNA sequences as clinical biomarkers were as follows:

- Reproducible, preferentially quantitative measurement is important in all clinical applications.

- Absolute methylation measurement (% methylation at individual sites) is more amenable to precise quantitation.
- Individual gene methylation measurement will likely be clinically useful in cancer detection, diagnosis and prognosis and classification and possibly in risk assessment.
- The performance of a biomarker is highly dependent on the choice of methylation detection method.

Choice of technology recommendations were:

- Bisulfite sequencing is optimal for the analysis of CpG island methylation of new genes
- Pyrosequencing is optimal for quantitation of individual CpG sites.
- Quantitative methylation-specific PCR is optimal for sensitive detection of methylated alleles.

Standardization issues and recommendations were:

- Different genes used in detection assays: establish optimal gene panel by interlaboratory testing.
- Different area of promoter of same gene: establish optimal gene panel by interlaboratory testing.
- Different technology used for analysis of methylation status: establish by interlaboratory testing of aliquots from universal standard.
- Different reference or controls used with same technology: establish by interlaboratory testing of aliquots from universal standard.

MicroRNA Expression Profiling for Diagnosis of Human Cancers

Although their function is not well understood, miRNAs control gene activity and play a major role in the development of human cancers. Bead-based flow cytometric expression profiling of miRNAs in samples from multiple human cancers has shown distinctive miRNA fingerprints. Generally there was downregulation of miRNAs in tumors compared with normal tissues. The miRNA profiles reflect the developmental lineage and differentiation state of the tumors and enabled successful classification of poorly differentiated tumors whereas mRNA profiles were highly inaccurate when applied to the same samples. These findings highlight the potential of miRNA profiling in cancer diagnosis and to select the most appropriate treatment.

MUC4 as a Diagnostic Biomarker in Cancer

Mucins are high molecular mass glycoproteins whose role in diagnosis, prognosis and therapy is being increasingly recognized owing to their altered expression in a variety of carcinomas. MUC4, a membrane-bound mucin encoded by a gene located on chromosome locus 3q29, is aberrantly expressed in several cancers including those of the bile duct, breast, colon, esophagus, ovary, lung, prostate, stomach and pancreas.

MUC4 expression pattern have potential use in the diagnosis and prognosis of various cancers (Chakraborty et al. 2008). MUC4 expression is a specific biomarker of epithelial tumors and its expression correlates positively with the degree of differentiation in several cancers. MUC4 has emerged as a specific biomarker of dysplasia, being expressed in the earliest dysplastic lesions preceding several malignancies, including pancreatic cancer. The presence of MUC4-specific antibodies in the serum and of the transcript in peripheral blood mononuclear cells of cancer patients may lead to a biomarker-based test for bedside application in high-risk individuals and those with established cancer.

Applications of Biomarkers for Cancer Diagnosis and Therapy

Role of biomarkers is increasingly promising with new targeted therapies for cancer, suggesting an integrated approach using the genetic make-up of the tumor and the genotype of the patient for treatment selection and patient management. The effectiveness of the Bcr-Abl kinase inhibitor imatinib (Novartis' Gleevec) in chronic myeloid leukemia (CML) and in a subset of patients with acute lymphoblastic leukemia (ALL) reduces with advancing disease and/or the development of resistance to imatinib. Tasigna® (Novartis) inhibits proliferation of hematopoietic cells expressing the mutants in Ph+ CML and ALL and is also effective against several imatinib-resistant Bcr-Abl mutants; it is combined with a battery of tests to define which patients should receive it.

Biomarkers can aid in patient stratification (risk assessment), treatment response identification (surrogate markers), or in differential diagnosis (identifying individuals who are likely to respond to specific drugs). To be clinically useful, a biomarker must favorably affect clinical outcomes such as decreased toxicity, increased overall and/or disease-free survival, or improved quality of life. Once the methods for assessment of the biomarker are established and the initial results show promise with regard to the predictive ability of a biomarker, it may be possible to achieve the goal of 'predictive oncology'. New drugs in oncology are being pursued with parallel development of a biomarker-based diagnostic test. As genotyping of drug-metabolizing enzymes becomes more widespread in the future, more changes are expected in drug labels.

Modern tumor pathology is now viewed at the molecular level ranging from immunohistochemistry (IHC) biomarkers, to gene signature classifiers and gene mutations, all of which provide crucial information about which patients will respond to targeted therapy regimens. An excellent review discusses the general types of targeted therapies used in a clinical setting and provides a short background on IHC, gene expression and DNA sequencing technologies (Tobin et al. 2015). The authors also highlight several strategies that are pivotal to the successful development of targeted anticancer drugs. Table 13.4 shows cancer biomarkers used for diagnosis and therapy.

Table 13.4 Cancer biomarkers used for diagnosis and therapy

Cancer type	Biomarker	Method of detection	Targted treatment
Advanced melanoma	CTLA4	IHC	Ipilimumab
Acute lymphoblastic leukemia	Philadelphia chromosome-positive BCR-ABL fusion	Karyotyping, FISH, or qPCR	Ponatinib, dasatinib, imatinib
Breast cancer	HER2 positive expression, ER- negative (HER2 overexpression subtype)	IHC, CISH, FISH	Trastuzamab, ado-trastuzumab emtansine, lapatinib, pertuzumab
	ER-positive, HER2 negative	IHC	Palbociclib, tamoxifen
	ER-positive, HER2 positive	IHC	Tamoxifen
	ER and HER2 negative (triple negative)	IHC	Olaparib
Cytogenic myeloid leukemia	Philadelphia chromosome-positive BCR-ABL fusion	Karyotyping, FISH, or qPCR	Bosutinib, nilotinib
Cytogenic myeloid leukemia	KIT mutation	qPCR	Imatinib
Cytogenic myeloid leukemia	BCR-ABL T315I mutation	qPCR	Ponatinib
Colorectal cancer (CRC)	KRAS mutation	IHC, qPCR	–
CRC	EGFR mutations	IHC, qPCR	Panitumumab
Gastrointestinal stromal tumors (GIST)	PDGFR	qPCR, FISH	Imatinib
GIST	KIT mutation	qPCR	Imatinib
Head & neck squamous cell carcinoma	EGFR mutations	IHC, qPCR	Cetuximab
Hodgkin lymphoma	PD-1 overexpression	IHC	Pembrolizumab
Melanoma	BRAF V600 mutation	qPCR	Vemurafenib
Metastatic melanoma	PD-1 overexpression	IHC	Nivolumab
Non-small cell lung cancer (NSCLC)	PD-1 overexpression	IHC	Nivolumab
NSCLC	PD-1	IHC	Atezolizumab
NSCLC	EGFR mutations	IHC, qPCR	Gefitinib, erlotinib
NSCLC	ALK rearrangement (EML4-, KIF5B- and TFG-ALK fusions)	FISH, IHC	Ceritinib, Crizotinib
Ovarian cancer	BRCA1/2 mutation/loss	IHC	Olaparib
Renal cell carcinoma	PD-1 overexpression	IHC	Nivolumab
Urothelial carcinoma	PD-1	IHC	Atezolizumab

ARTS as a Biomarker as Well as a Basis of Anticancer Drugs

Inhibitor of Apoptosis Proteins (IAPs) are frequently overexpressed in tumors and have become promising targets for developing anticancer drugs. IAPs can be inhibited by natural antagonists, but a physiological requirement of mammalian IAP antagonists remains to be established. Deletion of the mouse Sept4 gene, which encodes the IAP antagonist ARTS, a cell death protein found in every cell in the body, was shown to promote tumor development (García-Fernández et al. 2010). Sept4-null mice have increased numbers of hematopoietic stem and progenitor cells, elevated XIAP protein, increased resistance to cell death, and accelerated tumor development in an Eμ-Myc background. These phenotypes are partially suppressed by inactivation of XIAP. These results suggest that apoptosis plays an important role as a frontline defense against cancer by restricting the number of normal stem cells.

Apoptosis triggers ARTS and degrades apoptosis inhibitors. An approach to cancer therapy is based on replacing ARTS, which is lost in cancer, e.g. 75% of cells of bone marrow in leukemia lack ARTS. Lack of ARTS also makes cancer stem cells resistant to treatment. Scientists at Haifa University in Israel have developed a small molecule (peptide) ARTS that has been shown to enter cultured cancer cells and kill them selectively. Increasing expression of ARTS upto 5X does not kill normal cells. Personalized approach to cancer therapy can be developed by identifying patients who lack ARTS and treating them with ARTS-based therapy.

Asparagine Synthetase as Biomarker for Therapy with L-Asparaginase

L-Asparaginase (l-ASP), a bacterial enzyme used to treat acute lymphoblastic leukemia (ALL), selectively starves cells that cannot synthesize sufficient asparagine for their own needs. Studies show that cancer cells that contain less asparagine synthetase (ASNS) are more susceptible to L-ASP. The response to L-ASP therapy is often better when the expression of ASNS is limited. However, there is conflicting data from patient samples with regard to correlation between ASNS mRNA content and ASNase sensitivity. A study has shown that it is important to measure ASNS protein rather than mRNA in predicting ASNase responsiveness (Su et al. 2008).

L-ASP activity can be enhanced by combining it with antagonists of ASNS, such as siRNAs, antisense nucleotides, antibodies or small-molecule inhibitors for treatment of cancer. Reducing or suppressing the expression of ASNS potentiates the growth inhibitory activity of L-ASP four- to five-fold. Tissue microarrays have shown low ASNS expression in a subset of clinical ovarian cancers as well as other tumor types. Overall, such pharmacogenomic/ pharmacoproteomic studies suggest the use of L-ASP for personalized treatment of a subset of ovarian cancers (and perhaps other tumor types), with ASNS as a biomarker for selection of patients most likely to respond to L-ASP treatment. The technology is currently in the preclinical stage of development. With respect to L-ASP treatment of patients with solid tumors, phase I clinical trials have been initiated using L-ASP in combination with gemcitabine.

Peptide-Based Agents for Targeting Cancer Biomarkers

Small peptide-based agents have attracted wide interest as cancer-targeting agents for diagnostic imaging and targeted therapy. Efforts are being made to develop new high-affinity and high-specificity peptidomimetic or small-molecule ligands against cancer cell surface receptors. A high-affinity peptidomimetic ligand (LLP2A) against α4β1 integrin has been identified using both diverse and highly focused one-bead-one-compound combinatorial peptidomimetic libraries in conjunction with high-stringency screening. LLP2A can be used to image α4β1-expressing lymphomas with high sensitivity and specificity when conjugated to a near infrared fluorescent dye in a mouse xenograft model. Thus, LLP2A provides an important tool for noninvasive monitoring of α4β1 expression and activity during tumor progression, and it shows great potential as an imaging and therapeutic agent for α4β1-positive tumors.

PI3K Mutations as a Biomarker for use as a Companion Diagnostic

Variation in the PI3K gene could be a key biomarker for use as a companion diagnostic with certain cancer treatments. Several studies suggest that mutations in the PI3K oncogene are predictive for the success of certain treatments of patients suffering from lung, breast, colorectal and other cancers. QIAGEN has an active PI3K assay development and partnering program with pharmaceutical companies to develop and market tests based on this for new cancer drug candidates.

Biomarkers for Assessing Efficacy of Cancer Therapy

The high incidence of resistance to DNA-damaging chemotherapeutic drugs and severe side effects of chemotherapy have led to a search for biomarkers able to predict which patients are most likely to respond to therapy.

ERCC1-XPF Expression as a Biomarker of Response to Chemotherapy

ERCC1-XPF nuclease is required for nucleotide excision repair of DNA damage by cisplatin and related drugs, which are widely used in the treatment of cancer. The levels of ERCC1-XPF in a tumor could indicate whether it will be sensitive or resistant to a certain chemotherapeutic agent. Although several commercially available antibodies are suitable for immunodetection of ERCC1-XPF in some applications, only a select subset is appropriate for detection of this repair complex in fixed specimens. A study provides reliable tools for clinicians to measure the enzyme ERCC1-XPF as a biomarker in clinical specimens that could help stratify patients according to cancer risk, response to treatment and overall prognosis (Bhagwat et al. 2009).

P53 Expression Level as Biomarker of Efficacy of Cancer Gene Therapy

Advexin (Introgen Therapeutics) is a gene based drug, injected directly into tumors, which uses an adenoviral vector to deliver the wild-type p53 gene to tumor cells. Patients with advanced squamous cell carcinoma of the head and neck cancer, whose pretreatment tumor samples over-expressed p53, were significantly more likely to respond to Advexin therapy than those whose tumor showed little p53 protein. FDA has agreed to the use of Introgen's p53 molecular biomarkers in the analysis of Advexin clinical data used in support of submissions for approval. In updated data from phase II clinical trials, the predictive abnormal p53 biomarker was associated with a statistically significant increase in tumor responses to Advexin therapy. A reduction in tumor size was observed in 40% of patients with the abnormal p53 biomarker compared to none (0%) of the patients with p53 normal tumors (Nemunaitis et al. 2009). This makes p53 the first predictive biomarker test for a gene-based drug. Not only is this a way to predict if the gene therapy is likely to succeed, the patients for which it does work are the most difficult ones to treat. Accumulation of p53 corresponds with a poor response to traditional therapies such as radiation and chemotherapy, as well as lower survival and a shorter time to disease progression. Advexin has also achieved 100% response when combined with chemotherapy to treat locally advanced breast cancer, and a 69% response when used with radiation to treat non-small cell lung cancer.

Biomarkers of Angiogenesis for Developing Antiangiogenic Therapy

Angiogenesis, the formation of new blood vessels, is associated with normal physiological processes such as wound healing, ovulation or menstruation as well as with many diseases, such solid tumors. Advances in the pathomechanisms of tumor angiogenesis have led to new therapeutic options in the treatment of malignant tumors. During the development of antiangiogenic drugs, reports ranged from curing cancer to completely ineffective drugs. Some antiangiogenic agents have been approved and others are still in development. Many antiangiogenic drugs may encounter problems during clinical trials because they cannot reduce tumor size rapidly like chemotherapies can. This is another reason why biomarkers are needed for determining the effectiveness of antiangiogenic drugs in an early stage. Currently, there is a need to identify biomarkers that can both indicate biological activity and predict efficacy at the molecular level for antiangiogenesis drugs which are anticipated to result in tumor stasis rather than regression.

Biomarkers of Response to Antiangiogenic Agents

In order to identify biomarkers of response, athymic mice bearing L2987 human tumor xenografts were treated with the antiangiogenic agent brivanib alaninate (Bristol-Myers Squibb Co), which is currently under clinical evaluation (Ayers et al. 2007).

This is an orally available and selective tyrosine kinase inhibitor that targets the key angiogenesis receptors VEGFR-2 and FGFR-1. For these studies, tumor samples were collected from the xenografts and RNA was extracted for gene expression profiling on Affymetrix 430A mouse GeneChips. Statistical analysis was done using a defined set of genes identified to be coexpressed with VEGFR-2 from a clinical tumor gene expression profiling database and between tumor samples isolated from brivanib alaninate-treated and untreated mice. Tyrosine kinase receptor 1 (Tie-1), collagen type IVα1 (Col4a1), complement component 1, q subcomponent receptor 1 (C1qr1), angiotensin receptor–like 1 (Agtrl1), and vascular endothelial-cadherin (Cdh5) were all identified to be significantly modulated by treatment with brivanib alaninate. These genes, which may be potentially useful as biomarkers of brivanib alaninate activity, were further studied at the protein level in human colon tumor xenograft models, HCT116 and GEO, using immunohistochemistry-based approaches.

Circulating Endothelial Cells as Targets for Antiangiogenic Drugs

Previous studies have shown that the blood circulation of cancer patients has an abnormally high number of endothelial cells, which help construct blood vessels including those that feed the cancerous tumor. In addition to growing them directly from nearby blood vessels, tumors can also signal the body's bone marrow to boost the supply of endothelial cells in the blood circulation. Antiangiogenic drugs might combat cancer by preventing immature cells in the bone marrow from developing into endothelial cells. According to NCI researchers, if an antiangiogenic drug is successfully starving a cancer patient's tumor to death, the number of circulating endothelial cells (CECs) in the individual's bloodstream will decrease, thus providing a potential biomarker for gauging the medication's effectiveness.

Antiangiogenic drugs inhibit blood vessel development at the tumor by killing the endothelial cells lining tumor blood vessels and/or cutting off the supply of endothelial cells from bone marrow. These drugs are typically paired with chemotherapy agents. Unlike antiangiogenic drugs, chemotherapy agents directly attack tumor cells, and a reliable therapeutic biomarker for evaluating these agents is whether there are fewer cells in the tumor, or more, or just the same amount as before the treatment. Some chemotherapy drugs also have the benefit of being toxic to endothelial cells, providing a possible second biomarker for those agents.

Imaging Biomarkers for Evaluation of Antiangiogenic Agents

Some of the challenges posed by evaluation by imaging of angiogenesis inhibitors in phase I/II clinical trials. Because they reduce tumor growth or prevent metastases through primarily cytostatic modes of action – selectively inhibiting membrane receptors, cell cycle regulators or other signaling pathways – conventional end points based on reduction in tumor size may be inadequate for evaluating clinical response. Alternative imaging biomarkers of angiogenesis are being sought, which

can serve as early indicators of drug activity in clinical trials and may facilitate early pharmacodynamic assessments by speeding up the go/no-go decision-making process Dynamic contrast-enhanced MRI (DCE-MRI) is now frequently used in early clinical trial assessment of antiangiogenic and vascular disrupting compounds. Evidence of drug efficacy and dose-dependent response has been demonstrated by use of DCE-MRI with some angiogenesis inhibitors (O'Connor et al. 2007). Validation against histopathology biomarkers such as microvascular density is problematic in DCE-MRI, where micrometer scale biopsy changes must be compared against voxel resolution in millimetres. Nonetheless, histopathology validation is important and can substantiate the use of a biomarker in phase I/II trials. Both animal models and clinical studies are likely to be required to achieve comprehensive validation

In contrast to identifying the maximal tolerable dose, determination of the optimal biological dose, i.e. reaching biological activity at lower doses, has become the main target in the early development of antiangiogenic agents. This has been evaluated by different biomarker techniques. As a new standard in antitumor treatment, a better understanding of imaging in the treatment monitoring for antiangiogenic agents is important. Studies of tumor angiogenesis by tissue sampling rely on invasive procedures, adequate sampling and painstaking estimation of histological microvessel density. Attempts to develop wound healing assays to correlate angiogenesis in wounds with angiogenesis in tumor have been made but are still considered invasive and correlation of healthy with malignant tissue is still of limited validity. Several soluble biomarkers of tumor angiogenesis have been detected in various malignant diseases and have been evaluated for their use as surrogate markers in tumor angiogenesis. Soluble biomarkers were further investigated for use as imaging tools. Combination of biomarkers and imaging techniques has become an important method for developing anticancer drugs, an individual patient's response and monitoring of success of therapy at an early stage. Thus, time-consuming delays due to anatomy-based restaging procedures can be avoided. Characterization of soluble biomarkers can be combined with different imaging techniques such as ultrasound, CT, MRI and PET.

Tumor Endothelial Biomarkers

Lack of cancer-specific endothelial biomarkers has hindered the development of cancer therapies targeted against angiogenesis. Although the ability of Avastin to prolong survival in a phase III clinical trial of human colorectal cancer has established the validity of the antiangiogenic approach, realization of the full potential of a vascular targeting strategy may require the exploitation of molecules which are highly restricted in expression to tumor endothelium.

Technological advances in cellular fractionation and genomics have enabled the identification of several biomarkers preferentially expressed on human tumor endothelium. Tumor endothelial markers (TEMs) have the potential as new targets for cancer therapy. Studies of these TEMs are expected to aid in our understanding of angiogenesis and could lead to the development of new imaging and diagnostic

agents for cancer. Some of the secreted TEMs can serve as surrogate biomarkers in the determination of the optimum biological dose for the current antiangiogenic drugs in clinical trials.

VEGF Signaling Inhibitors as Biomarkers

A systematic review using PubMed, MEDLINE and American Society of Clinical Oncologist (ASCO) databases was conducted for articles (including abstracts) presented in 2007–2009 to compare undertaken new small-molecule tyrosine kinase inhibitors with VEGF receptor as one of their targets (Wood et al. 2009). Factors considered included mode of action (targets), toxicity and usefulness of biomarker data. Search terms included 'angiogenesis inhibitors', 'tyrosine kinase inhibitors', 'VEGF' and 'biomarkers'. Nine compounds were selected for detailed comparison. The toxicity profiles of the compounds were similar. Many exposure biomarkers were identified that helped to determine the dose and scheduling of these compounds in clinical trials. Progress has also been made in identifying potential efficacy and predictive biomarkers for these new agents; however, these are yet to be validated.

VEGF-PET Imaging for Analysis of Angiogenic Changes within a Tumor

Non-invasive imaging of angiogenesis could ease the optimization of antiangiogenesis treatments for cancer. A study has evaluated the role of VEGF-PET as a biomarker of dynamic angiogenic changes in tumors following treatment with the kinase inhibitor sunitinib (Nagengast et al. 2010). The effects of sunitinib treatment and withdrawal on the tumor was investigated using the new VEGF-PET tracer ^{89}Zr-ranibizumab as well as ^{18}F-FDG PET, and ^{15}O-water PET in mouse xenograft models of human cancer. The imaging results were compared with tumor growth, VEGF plasma levels and immunohistologic analyzes. In contrast to ^{18}F-FDG and ^{15}O-water PET, VEGF-PET demonstrated dynamic changes during sunitinib treatment within the tumor with a strong decline in signal in the tumor center and only minimal reduction in tumor rim, with a pronounced rebound after sunitinib discontinuation. VEGF-PET results corresponded with tumor growth and immunohistochemical vascular- and tumor- biomarkers. These findings highlight the strengths of VEGF-PET imaging to enable serial analysis of angiogenic changes in different areas within a tumor.

Biomarkers of Prognosis in Cancer Treatment

Various biomarkers of prognosis in cancer treatment have been investigated and some novel ones are shown in Table 13.5. Prognosis is considered in more detail along with biomarkers of individual cancers.

Table 13.5 Novel biomarkers of prognosis in cancer treatment

Biomarker	Role in cancer	Findings in cancer	Prognostic significance
PI3K/an enzyme that helps control cell growth	Mutations in PIK3CA, the gene encoding the catalytic subunit of PI3K, activate the PI3K-AKT-mTOR pathway in cancer cells	PIK3CA mutations are frequent in cancers of endometrium, ovaries, and breast	In phase I clinical trials, 40% of patients with mutations in PI3K gene responded to treatment targeting PI3K signaling
CYP2D6/ role in metabolizing several drugs	Polymorphisms of the gene may interfere with action of anticancer drugs	Polymorphisms of CYP2D6 and co-administration of drugs that inhibit CYP2D6 reduce plasma levels of endoxifen, the active metabolite of tamoxifen, in patients being treated for breast cancer	Patients who only took CYP2D6-inhibiting medications in conjunction with tamoxifen had worse time to progression and worse overall survival compared to patients who did not take CYP2D6-inhibitors
TIMP-1/ tissue inhibitor of matrix metalloproteinase 1	TIMP-1 is over-expressed in many cancers	TIMP-1 is associated with poor outcome in renal cell carcinoma (RCC)	Sorafenib (a multikinase inhibitor) phase III TARGET trial showed that TIMP-1 is an independent prognostic biomarker for survival in RCC
Trim62/a novel protein biomarker	Trim62 regulates p27 stability but also its localization in HER2 positive breast cancers. p27 in the nucleus functions as a tumor suppressor but, when in the cytoplasm, it may enhance metastasis.	Trim62 is responsible for the elevated cytoplasmic p27 in HER2 positive breast cancer tumors	Trim62 could be a potential biomarker to predict patient response to anti-HER2 therapeutics such as lapatinib

Abbr: TARGET = Treatment Approaches in Renal Cancer Global Evaluation Trial

Biomarkers for Monitoring Cancer Therapy

Tumor biomarkers that are measured during postoperative surveillance are also frequently used for monitoring patients with advanced cancer receiving systemic therapy. Examples are; CEA for colorectal cancer, CA 15-3 for breast cancer, and AFP for hepatocellular cancer. Usually decreasing levels of biomarkers following the initiation of therapy correlates with tumor regression and increasing levels predict progressive disease. Tumor biomarkers, however, should not be sole criteria for assessing response to therapy. A caveat in the use of biomarkers for monitoring therapy in patients with advanced cancer is that transient increases or spikes may occur within the first few weeks of start of therapy, which appear to be due to tumor cell necrosis or apoptosis in response to the initial treatment with chemotherapy (Duffy 2013). Such transient increases have not yet been reported with biological therapies such as therapeutic antibodies, e.g. Herceptin, cetuximab and panitumumab.

Biomarkers of Drug Resistance in Cancer

Human cancers are mostly found to be resistant to therapy at the time of drug presentation (primary responses), tumors being intrinsically drug resistant (innate or de novo drug resistance). Only a few become resistant after an initial response (acquired responses), the tumors developing resistance to chemotherapy during treatment (acquired drug resistance). In the latter group, a tumor cell may express drug resistance by combining several distinct mechanisms induced by its exposure to various drugs. In the former group, however, this is unlikely to be the case.

Pharmacogenetics and pharmacogenomics studies of the relationship between individual variations and drug response rates reveal that genetic polymorphisms of specific genes is associated with clinical outcomes in patients treated through chemotherapy, and amplification of genes encoding drug targets or transporters alters the sensitivity of cancer cells to a particular chemotherapy. Loss of heterozygosity (LOH) at specific regions of chromosomes has been identified in specific cancers but its effect on treatment outcome remains controversial.

A Systems Approach to Biomarkers of Innate Drug Resistance

Comparison of gene expression patterns obtained with microarrays on tumor samples from cancer patients prior to drug exposure and after development of resistance to anticancer therapy may provide an insight into molecular mechanisms of drug resistance. The information generated from such studies might also provide new biomarkers for prediction of the chemo-sensitive and -resistant states to the combined chemotherapy in newly diagnosed cancer patients, enabling therapeutic

adjustment. Further integration with data collected at the genomic level through mutation analysis, at the level of the entire transcriptome by complementary comprehensive methods such as massive parallel signature sequencing, at the proteome level with emerging global technologies such as ICAT coupled with MS, and at the metabolome level by MS or NMR should provide the basis for designing reliable predictive biomarkers and deciphering the molecular pathways involved in drug responses.

Epithelial Membrane Protein-1 as a Biomarker of Gefitinib Resistance

Gefitinib is a small-molecule inhibitor that competes for the ATP-binding site on epithelial growth factor receptor (EGFR) and has been approved for patients with advanced lung cancers. Treatment with gefitinib has resulted in clinical benefit in patients, and, recently, heterozygous somatic mutations within the EGFR catalytic domain have been identified as a clinical correlate to objective response to gefitinib. However, clinical resistance to gefitinib limits the utility of this therapeutic to a fraction of patients, and objective clinical responses are rare. Epithelial membrane protein-1 (EMP-1), an adhesion molecule, has been identified as a surface biomarker whose expression correlates with acquisition of gefitinib resistance. EMP-1 expression further correlates with lack of complete or partial response to gefitinib in lung cancer patient samples as well as clinical progression to secondary gefitinib resistance. EMP-1 expression and acquisition of gefitinib clinical resistance is independent of gefitinib-sensitizing EGFR somatic mutations. There is a probable cross-talk between this molecule and the EGFR signaling pathway.

Methylation Biomarkers of Drug Resistance in Cancer

The association of the methylation status of DNA repair genes such as O(6)-Methylguanine-DNA Methyltransferase (MGMT) and MLH1 illustrate the two main mechanisms of response to DNA damaging agents. Loss of methylation of MGMT, and the subsequent increase in gene expression, leads to a reduction in response to alkylating agents as a result of enhanced repair of drug-induced DNA damage. Conversely, the increase in methylation of MLH1 and its resulting loss of expression has been consistently observed in drug-resistant tumor cells. MLH1 encodes a mismatch repair enzyme activated in response to DNA damage; activation of MLH1 also induces apoptosis of tumor cells, and thus loss of its expression leads to resistance to DNA-damaging agents. Other methylation-regulated genes that could serve as biomarkers in cancer therapy include drug transporters, genes involved in microtubule formation and stability, and genes related to hormonal therapy response. These methylation biomarkers have potential applications for disease prognosis, treatment response prediction, and the development of novel treatment strategies for cancer.

STAT3 and Resistance to Cisplatin

STAT3 (Signal transducer and activator of transcription 3) seems to play crucial roles in cell proliferation and survival, angiogenesis, tumor-promoting inflammation, and suppression of antitumor host immune response in the tumor microenvironment. STAT3 is central to determining the type of inflammation that can either promote or inhibit tumor development, direct inhibition of STAT3 may represent a promising therapeutic target to reprogram tumor-promoting inflammation into tumor-suppressing inflammation (Kato 2011). STAT3 is elevated in ~82% of head and neck cancers and has been associated with cisplatin resistance. STAT3 inhibitors such as FLLL32 (a compound based on curcumin) may be useful adjuvants to cisplatin to overcome drug resistance (Abuzeid et al. 2011).

Biomarkers of Radiation Therapy for Cancer

Radiation therapy is used to treat half of all cancer patients. Response to radiation therapy varies widely among patients. Proteomic profiling strategies may be helpful in cancer before and during radiotherapy in an effort to discover clinical biomarkers of radiation exposure. High-resolution SELDI-TOF MS has been used to generate high-throughput proteomic profiles, which can be analyzed for unique biomarker signatures using supervised classification techniques. MS-based protein identification on pooled sera may reveal specific protein fragments that are altered with radiation exposure. Computer-based analyses of the SELDI protein spectra can distinguish unexposed from radiation-exposed patient samples with 91% to 100% sensitivity and 97% to 100% specificity using various classifier models. Using direct identity techniques of albumin-bound peptides, known to underpin the SELDI-TOF fingerprints, unique protein fragments/peptides have beendetected in the radiation exposure group, including an IL-6 precursor protein. The composition of proteins in serum seems to change with ionizing radiation exposure.

A combination of genome-wide association study (GWAS), gene expression, radiation response, and gene knockdown has narrowed in on five genes that seem to be associated with radiation response in both lymphoblastoid cell lines and specific cancer cell lines (Niu et al. 2010). Functional validation using siRNA knockdown in multiple tumor cell lines showed that C13orf34, MAD2L1, PLK4, TPD52, and DEPDC1B each significantly altered radiation sensitivity in at least two cancer cell lines. Such studies can help to identify novel biomarkers that might contribute to variation in response to radiation therapy and enhance our understanding of mechanisms underlying the variation in response to radiation. That might help to maximize radiation efficiency in the tumor while minimizing side effects in normal tissues. The work may ultimately lead to biomarkers for individualizing radiation therapy based on the expression of these candidate genes and may make it possible to design novel combination therapy for selected patients based on these biomarkers to overcome resistance.

Role of Biomarkers in Drug Development in Oncology

Response evaluation criteria in solid tumors (RECIST) guidelines have been in use for evaluation of results of treatment of cancer in clinical trials since 2000. RECIST is a combined assessment of all existing lesions, characterized by target lesions (to be measured) and nontarget lesions, which is used to extrapolate an overall response to treatment. There are some limitations to this approach. For example, trial eligibility and end points based solely on tumor regression are not applicable to the majority of the clinical manifestations of prostate cancers representing all clinical states. PSA-based eligibility and outcomes under RECIST conflict with established reporting standards for the states of a rising PSA and castrate metastatic disease. The clinical manifestations of prostate cancer across multiple disease states are not addressed adequately using the eligibility criteria and outcomes measures defined by RECIST. Treatment effects can be described more precisely if eligibility criteria are adapted to the clinical question being addressed and clinical state under study, focusing on the duration of benefit defined biochemically, radiographically, and/or clinically.

Several candidate biomarkers have the potential to serve as a guide the development of safer and more efficacious drugs. Challenges faced during biomarker discovery as well as during technology and process translation have been reviewed with specific references to protein biomarkers for cancer drug development (Lee et al. 2007).

Biomarkers will provide a better dimension of assessment of response to treatment. Various clinical areas are expected to move toward adoption of biomarkers at different rates but oncology is expected to have the largest gains from biomarkers over the next five to ten years. The reasons for this are as follows:

- Cancer is a genetic disease.
- Cancer specimens are readily available for testing.
- Cancer is life-threatening, creating an urgency to find a treatment solution and a greater willingness to accept risk.
- Patients are very sick and often receive a number of drugs in a short time.
- Patients have low clinical response rates to treatment.

Biomarkers hold promise for optimization in dosing, adverse event prediction, efficacy evaluation, lead prioritization, and mechanism-of-action profiling of drug candidates. In addition, oncology has a strong pipeline of drugs in development and a number of smaller companies engaged in innovative research. Already, physicians have access to tests that help identify breast cancer patients at low risk for recurrence, who may not benefit from systemic adjuvant therapy. Identifying suitable biomarkers of biologic activity is important when assessing novel anticancer compounds such as angiogenesis inhibitors to optimize the dose and schedule of therapy.

Molecular Imaging of Tumor as a Guide to Drug Development

The field of molecular imaging offers potential to deliver a variety of probes that can image noninvasively drug targets, drug distribution, cancer gene expression, cell surface receptor or oncoprotein levels, and biomarker predictors of prognosis, therapeutic response, or failure. Some applications are best suited to accelerate preclinical anticancer drug development, whereas other technologies may be directly transferable to the clinic. Efforts are underway to apply noninvasive in vivo imaging to specific preclinical or clinical problems to accelerate progress in the field. By enabling better patient selection and treatment monitoring strategies, molecular imaging will likely reduce the future cost of drug development in oncology.

For decades anatomic imaging with CT or MRI has facilitated drug development in medical oncology by providing quantifiable and objective evidence of response to cancer therapy. In recent years metabolic imaging with ^{18F}fluorodeoxyglucose-PET has added an important component to the oncologist's armamentarium for earlier detection of response that is now widely used and appreciated. These modalities along with ultrasound and optical imaging (bioluminescence, fluorescence, near-infrared imaging, multispectral imaging) have become used increasingly in preclinical studies in animal models to document the effects of genetic alterations on cancer progression or metastases, the detection of minimal residual disease, and response to various therapeutics including radiation, chemotherapy, or biologic agents.

Use of PET to Assess Response to Anticancer Drugs

Thymidylate synthase is a key enzyme for the de novo synthesis of DNA and as such a target for anticancer drug development. ^{18F}FLT-PET can be used to measure thymidylate synthase inhibition as early as 1 to 2 hours after treatment with 5-FU by a mechanism involving redistribution of nucleoside transporters to the plasma membrane. FDA collaborates with NCI to determine the role of PET scans in clinical trials to assess whether a patient's tumor is responding to treatment even if it is not shrinking. Only PET can determine if tumor cells are undergoing apoptosis and the drug is having the desired effect. Changes in tumor size may take a long time. PET technology can detect HER2 positivity as well as biomarkers of hypoxia (FMISO) and angiogenesis (integrin $\alpha\nu\beta3$ and VEGF). Specific molecular pathways may also be analyzed, e.g. for abnormal PI3-kinase/AKT activity, by measuring the accumulation of 11C acetate by PET, and that of the RAS/RAF/MEK/ERK pathway by changes in labeled choline.

Of the molecular biochemical alterations that occur during apoptosis, activation of caspases, notably caspase-3, is the most attractive for developing specific in vivo molecular imaging probes. An isatin-5 sulfonamide, ^{18F}ICMT-11, has subnanomolar

affinity for activated capsase-3, high metabolic stability, and binds to a range of drug-induced apoptotic cancer cells in vitro and in vivo by up to 2-fold at 24 h post-treatment compared to vehicle treatment (Nguyen et al. 2009). The increased signal intensity in tumors after drug treatment, detected by whole body in vivo microPET imaging, is associated with increased apoptosis. Thus ^{18F}ICMT-11, as a caspase-3/7 specific PET imaging radiotracer for the assessment of tumor apoptosis, is useful in anticancer drug development and the monitoring of early responses to therapy.

CT may not be optimally suitable for assessment of oncolytic virus treatments because of paradoxical inflammatory tumor swellings, which result from virus treatments, particularly when viruses are armed with immunostimulatory molecules. In a comparative study of patients treated by viral oncolysis, FDG-PET was more sensitive in detection of responses than tumor size determined by CT (Koski et al. 2013).

Use of MRI to Assess Response to Anticancer Drugs

Diffusion-weighted MRI (DWI) provides physiological imaging end-point since tumor necrosis and cellularity are seen early in response to anti-angiogenic treatment. Dynamic Contrast Enhanced (DCE)-MRI uses T2 captured images immediately after contrast injection (evaluating perfusion) and T1 images over a few minutes to examine extravasation of contrast for evaluating blood volume within tumor and microvascular permeability. Measurable changes in tumor perfusion or vascular permeability provide pharmacodynamic evidence of antiangiogenic effect and may provide information early in a treatment course about likely response to such therapies.. In addition, the vascular response measured using DCE-MRI seems to be a useful indicator of drug pharmacology, and additional research is needed to determine if it is a suitable biomarker for predicting clinical activity.

A balanced review of the state of the art of DWI and DCE-MRI discusses what these two quantitative MRI techniques can offer and what barriers remain before they can be readily and regularly incorporated into clinical trials (Yankeelov et al. 2011). Although there are a number of difficulties that need to be resolved, they are not insurmountable and these techniques will ultimately find their way into RECIST.

Biomarkers in Plucked Hair for Assessing Cancer Therapy

There is a need for a technology that can quantitatively assay multiple proteins from a single hair follicle while preserving the morphology of the follicle. For proteomic profiling, the technology should be less labor intensive, with a higher throughput, more quantitative and more reproducible than immunohistochemistry. Layered expression scanning of hair (LES-hair) has been used to detect the levels and localization of proteins in plucked hair follicles. These proteins included

cleaved caspase 3, Ki-67 and the phosphorylated forms of c-Kit, EGF receptor and VEGF receptor. LES-hair provides a research tool for studying the basic biology of plucked hair follicles and has potential clinical applications such as using plucked hair follicles as a surrogate tissue to monitor pharmacodynamic effects of targeted cancer therapies.

The "plucked hair" biomarker program (EpiStem Inc) has evolved from the discovery of the link between the stem cells in the small intestine and the hair follicle. This biomarker has been developed as a non-invasive tool to measure drug effects on adult epithelial stem cells and tissues. Plucked human hairs are analyzed for the corresponding changes in gene expression at various times during cancer treatment. Gene expression changes in hairs can provide pharmaceutical companies with a measure of drug exposure, toxicity, dose/schedule and patient selection in preclinical and clinical drug development. By comparing the gene sets linked to tumors as well as drug exposure and toxicity, it may be possible to eventually use the hair biomarker approach for evaluating the effectiveness of new cancer treatments. This approach also has the potential to offer oncologists a simple means to more effectively treat cancer patients.

Safety Biomarkers in Oncology Studies

In the conduct of clinical studies of anticancer agents administered to patients with advanced malignancy, safety biomarker results are playing an increasingly important role. Safety biomarkers may be appropriately used for decision making by the application of uniform criteria, especially in situations where there is a degree of correlation between biomarker changes and corresponding clinical outcomes. While new safety biomarkers have major value, their applications require careful consideration to avoid unintended consequences that could negatively affect patient care and the development of promising new oncology therapeutics.

Role of Biomarkers in Phase I Clinical Trials of Anticancer Drugs

A new model has been suggested of early clinical trial design involving patient selection through predictive biomarkers for selected molecularly targeted agents (Carden et al. 2010). This model can maximize the chances of patient benefit and the yield of biologic and clinical information as well as direct subsequent clinical trials. Ultimately, this may result in a new paradigm of drug development, focused on patients with tumors with the same oncogenic molecular abnormalities, rather than focused on patients with tumors from the same anatomical site or similar histopathology. Such biomarkers, predicting response to molecular targeted

agents, have the potential for selecting patients for these trials who are more likely to benefit from the treatment. This may facilitate early experience of and steps towards clinical qualification of predictive biomarkers, generate valuable information on cancer biology, and enable development of personalized anticancer therapy. New models of phase I study design of cancer that incorporate patient selection based on predictive biomarkers have the potential to accelerate anticancer drug development for many molecular-targeted novel agents. Indeed, it is probable that the early identification of such predictive biomarkers will improve the odds of eventual drug registration.

Scientists at Roche have used a genomics-based approach to identify pharmacodynamic biomarkers for a cyclin-dependent kinase inhibitory drug, R547, which is a potent cyclin-dependent kinase inhibitor with a potent antiproliferative effect at pharmacologically relevant doses and is currently in phase I clinical trials (Berkofsky-Fessler et al. 2009). Using preclinical data derived from microarray experiments, they identified pharmacodynamic biomarkers for further testing in blood samples from patients in clinical trials. The selection of candidate biomarkers was based on several criteria: relevance to the mechanism of action of R547, dose responsiveness in preclinical models, and measurable expression in blood samples. They identified 26 potential biomarkers of R547 action and tested their clinical validity in patient blood samples by quantitative real-time PCR analysis. Based on the results, eight genes (FLJ44342, CD86, EGR1, MKI67, CCNB1, JUN, HEXIM1, and PFAAP5) were selected as dose-responsive pharmacodynamic biomarkers for phase II clinical trials.

Met Receptors as Targets for Anticancer Drugs

Met receptor tyrosine kinase was discovered in 1984 as an oncogene. Thirty years later, Met and its ligand hepatocyte growth factor/scatter became promising targets for novel anticancer therapies, with more than 240 clinical trials conducted until end of 2014. A review has discussed research on Met receptor, which allowed moving this biomarker from bench to bedside (Furlan et al. 2014). It took three decades of basic research to unravel the structural basis of the ligand/receptor interaction and their complex downstream signaling network. During this period, animal models highlighted their crucial role in the development and homeostasis of epithelial organs. In parallel, involvement of Met in tumorigenesis was confirmed by the direct association of its deregulation to poor prognosis in cancer. On the basis of these data, pharmaceutical companies developed many Met inhibitors, some of which are in phase III clinical trials. These are impressive achievements, but the mechanism of resistances to Met-targeted therapies remains to be fully understood.

Biomarkers According to Organ/Type of Cancer

There is no general biomarker for cancer. Since the tumors involving different organs differ considerably, biomarkers have been investigated according to the type of cancer.

Bladder Cancer Biomarkers

Tumors arising from the urothelial mucosa lining the urinary bladder are the most common malignancy of bladder and upper urinary tract (ureters and renal pelvis). Proteomic techniques have been used to systematically identify the proteins in urine samples of patients with squamous cell carcinoma of the bladder, identifying a protein called psoriacin as an early biomarker for the disease.

A multitarget, multicolor FISH assay has been developed for the detection of urothelial carcinoma in urine specimens. A FISH assay containing centromeric probes to chromosomes 3, 7, and 17 and a locus-specific probe to band 9p21 has high sensitivity and specificity for the detection of bladder cancer from voided urine specimens. UroCor Inc., using Ambion's technology, has developed a test for direct identification of p53 gene mutations in patients with bladder cancer utilizing a urine specimen.

Detection of FGFR3 Mutations in Urine for Diagnosis of Bladder Cancer

Fibroblast Growth Factor Receptor 3 (FGFR3), an important and well-established DNA biomarker is present in 30–50% of patients with bladder cancer, but also correlates with a lower rate of recurrence. This is the basis of a non-invasive urine-based diagnostic test for bladder cancer. A small sample of urine is collected and is subjected to next generation sequencing (NGS) to determine the presence of mutations. NGS enables higher sensitivity than qPCR. The exact percentage of mutated DNA in body fluids is unknown although amount of 1% have been detected. Ultradeep NGS-based assays for FGFR3 can detect a mutation when it is present in as little as 0.02% of the total amount of DNA in urine (Millholland et al. 2012). However, this is not possible at POC and the urine specimen has to be sent to a lab that specializes in NGS.

NMP22 BladderChek

NMP22 BladderChek (Alere) is a point-of-care test for bladder cancer that returns results while the patient is in the doctor's office. Current tests performed in a central laboratory take 2–3 days to deliver results. NMP22 BladderChek measures the level

of NMP22, a nuclear matrix protein, in the urine. The test would be used in conjunction with cystoscopy, a procedure in which a fiberoptic tube is inserted into the bladder through the urethra permitting visual examination of the bladder.

Urinary Telomerase as Biomarker for Detection of Bladder Cancer

Telomerase is present in about 95% of all epithelial cancers and therefore has great potential as a cancer biomarker. Expression levels of human telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR) are analyzed by RT-PCR in urine samples from subjects with bladder cancer and controls with benign genitourinary diseases as well as healthy subjects. Quantitative urinary hTR analysis detects bladder cancer with an overall sensitivity of 77.0%, whereas hTERT analysis reaches a sensitivity of 55.2%. Both hTR and hTERT are significantly more sensitive than cytology. Quantitative hTR analysis is the most accurate telomerase-based test for bladder cancer detection and has the potential to replace cytology as a noninvasive biomarker for disease diagnosis and follow-up.

Concluding Remarks Abut Biomarkers of Urinary Cancer

In general, the best new biomarkers give higher sensitivity than urinary cytology, but specificity is usually lower. By using new biomarkers, the intervals between follow-up cystoscopies can be increased and the detection of relapse can be improved. But to date no non-invasive biomarker has proven to be sensitive and specific enough to replace cystoscopy, either for the diagnosis or for the follow-up of bladder cancer. However, biomarker combinations and algorithms for risk assessment hold promise for the future (Lintula and Hotakainen 2010).

Brain Tumor Biomarkers

The most common primary malignant tumor of the brain in adults is glioblastoma multiforme (GBM). Routine diagnosis is based on brain imaging and histological examination. In the past, most of the genetic studies of tumors involved cytogenetic analysis. Biomarkers are now used for various applications in brain tumors, e.g. to assess malignancy and guide therapy. In particular, the use of loss of heterozygosity (LOH) and methylation specific PCR (MSP) is used clinically in several centers. Molecular techniques, such as LOH testing, FISH, DNA sequencing and and O^6-methylguanine-DNA methyltransferase methylation status are currently being employed in assessment of gliomas in some laboratories. Table 13.6 shows biomarkers of brain tumors.

Table 13.6 Biomarkers of brain tumors

Biomarkers of gliomas
IL-16 IL-16 rs11556218 polymorphism as a susceptibility biomarker for glioma (Luo et al. 2014)
EGFR gene amplification and BRAF rearrangement detected by FISH (fluorescence in situ hybridization)
ELTD1 (EGF, latrophilin and 7 transmembrane domain-containing 1 on chromosome 1
Loss of heterozygosity (LOH) on chromosomes 1p, 19q, 17p and 10q
Methylation profiling of brain tumors
Detection of methylation-dependent DNA sequence variation: methyl SNP
Methylation of TMS1, an intracellular signaling molecule
MCJ as a biomarker of medulloblastoma
Protein biomarkers
Circulating microvesicles (exosomes) containing mRNA, miRNA and angiogenic proteins
CSF protein profiling: N-myc oncoprotein, caldesmon, attractin
Receptor protein tyrosine phosphatase
Serum protein fingerprinting
Biomarkers of angiogenesis in brain tumors
VEGF-R2 levels in tumor tissues: in vivo evaluation of angiogenesis using molecular MRI
Metabolite biomarkers detected by magnetic resonance spectroscopy
N-acetylaspartate (diminished)
Choline
Lactate
miRNA
Biomarkers of response to therapy
Biomarkers to predict response to EGFR inhibitors
MRI biomarker for response of brain tumor to therapy
Biomarkers of prognosis of glioblastoma multiforme (GBM)
14-3-3zeta positive expression

14-3-3zeta Positive Expression as a Prognostic Biomarker for GBM

A clinical study has shown that 14-3-3zeta positive expression was observed in approximately 74.5% of patients with GBM who had lower overall survival rates and median survival time than those in the 14-3-3zeta negative group (Yang et al. 2011). 14-3-3zeta positive expression in tumor cells also was correlated with a shorter interval to tumor recurrence. Univariate and multivariate analyses showed that 14-3-3zeta positive expression was an independent prognostic factor for GBM and can be used as a biomarker.

ALDH1A3 as a Biomarker of GBM

ALDH1A3 is a specific biomarker enzyme for mesenchymal glioma stem-like cells (GSCs), and among the heterogeneous mix of cells in a GBM tumor, cells with high levels of ALDH1A3 expression are more tumorigenic in vivo than are cells that are low in ALDH1A3 (Cheng et al. 2016). The authors also found that the FOXD1 transcription factor regulates the production of ALDH1A3 in mesenchymal GSCs. In clinical samples of GBM from patients, the expression levels of both FOXD1 and ALDH1A3 were inversely correlated with disease progression, i.e. gliomas with high levels were more rapidly fatal than were gliomas with low levels. GA11, a small molecule inhibitor of ALDH, inhibits proliferation of glioma spheres in cell culture, and inhibits xenograft growth of GBM in mouse brains. GA11 is a potential therapy for GBM and clinical trials are planned.

Biomarkers to Predict Response to EGFR Inhibitors

Epidermal growth factor receptors (EGFRs) are amplified and overexpressed in many different human cancers, a phenomenon generally associated with poor prognosis. Inhibitors of the tyrosine kinase activity associated with this receptor, e.g. erlotinib, have been approved for the treatment of chemotherapy-refractory NSCLC. Clinical trials have shown that patients with GBM whose tumors exhibit overexpression and amplification of EGFR respond better to erlotinib than patients who had normal levels of this gene and protein. The phosphorylation state of PKB/Akt is also an important determinant for response, with low phospho-PKB/Akt levels predicting good response to erlotinib. These data underscore the importance of placebo-controlled trials to distinguish between prognostic indicators of disease progression more generally and predictive biomarkers of response to therapy. Ultimately the goal of these studies is to allow selection of patients who will preferentially respond to EGFR inhibitors.

Biomarkers for Predicting Recurrence of Meningiomas

Meningiomas are usually benign and treated by surgical resection. The natural history of surgically treated intracranial meningiomas can be quite variable. Recurrence and patient outcome cannot currently be predicted with accuracy. A study has examined tumor hypoxia-regulated biomarkers, preoperative imaging, measures of proliferation, and angiogenesis in predicting patient outcome (Jensen and Lee 2012). Results show that hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF), and MIB-1 proliferation index are significantly correlated with tumor recurrence. With further study, these molecular biomarkers may be used to predict outcome for patients with intracranial meningiomas.

CD133 as Biomarker of Resistance to Radiotherapy

Cancer stem cells contribute to resistance of GBM to radiotherapy through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity. The fraction of tumor cells expressing CD133 (Prominin-1), a biomarker for both neural stem cells and brain cancer stem cells, rises after radiation in GBM. The radioresistance of CD133-positive glioma stem cells can be reversed with a specific inhibitor of the Chk1 and Chk2 checkpoint kinases. These results suggest that CD133-positive tumor cells represent the cellular population that confers GBM radioresistance and could be the source of tumor recurrence after radiation. Targeting DNA damage checkpoint response in cancer stem cells may overcome this radioresistance and provide a therapeutic model for GBM.

Circulating Microvesicles as Biomarkers

GBM tumor cells release microvesicles (exosomes) containing mRNA, miRNA and angiogenic proteins, which are taken up by normal host cells, such as brain microvascular endothelial cells. By incorporating an mRNA for a reporter protein into these microvesicles, it was demonstrated that messages delivered by microvesicles are translated by recipient cells (Skog et al. 2008). These microvesicles are also enriched in angiogenic proteins and stimulate tubule formation by endothelial cells. Tumor-derived microvesicles therefore serve as a means of delivering genetic information and proteins to recipient cells in the tumor environment. GBM microvesicles also stimulated proliferation of a human glioma cell line, indicating a self-promoting aspect. mRNA mutant/variants and miRNAs characteristic of gliomas could be detected in serum microvesicles of GBM patients. The tumor-specific EGFRvIII was detected in serum microvesicles from several glioblastoma patients and can be considered a biomarker. Thus, tumor-derived microvesicles represent a new way of obtaining information about a cancer without a biopsy, offering a means of choosing the best therapy, seeing how a patient responds to treatment, and possibly offering a way to deliver therapies to the tumor. Exosome Diagnostics Inc. has licensed the technology for further development.

CSF Attractin as a Biomarker of Malignant Astrocytoma

By use of proteomic techniques to identify secreted proteins in the CSF samples of patients with various CNS diseases, attractin is consistently found to be elevated in the samples of patients with malignant astrocytoma. Attractin is produced and secreted by the tumor cells as shown by IHC. Furthermore, it has been observed that CSF from brain tumor patients induces glioma cell migration and that attractin is largely responsible for this promigratory activity. Attractin, which is normally absent in the brain tissue, is not only an important biomarker of malignant astrocytoma, but may also be an important mediator of tumor invasiveness. It is a potential target for future therapies.

ELTD1 as a Biomarker of Gliomas

Advanced data mining and a novel bioinformatics method was used to predict ELTD1 (EGF, latrophilin and 7 transmembrane domain-containing 1 on chromosome 1) as a potential novel biomarker that is associated with gliomas (Towner et al. 2013). Validation was done with IHC to detect levels of ELTD1 in human high grade gliomas, and rat F98 glioma tumors. In vivo levels of ELTD1 in rat F98 gliomas were assessed using molecular MRI. ELTD1 was found to be significantly higher in high-grade gliomas compared to low-grade gliomas (21 patients), and compared well to traditional IHC markers including VEGF, GLUT-1, CAIX, and HIF-1α. ELTD1 gene expression indicates an association with grade, survival across grade, and an increase in the mesenchymal subtype. This study strongly suggests that associative analysis was able to accurately identify ELTD1 as a putative glioma-associated biomarker. The detection of ELTD1 was also validated in both rodent and human gliomas, and may serve as an additional biomarker for gliomas in preclinical and clinical diagnosis of gliomas.

Methylation Profiling of Brain Tumors

Tumorigenesis is characterized by alterations of methylation profiles including loss and gain of 5-methylcytosine. In each GBM, hundreds of genes are subject to DNA hypermethylation at their CpG island promoters. A subset of GBMs is also characterized by locus-specific and genome-wide decrease in DNA methylation. Other epigenetic alterations, such as changes in the position of histone variants and changes in histone modifications are also likely important in the molecular pathology of GBM. Alterations in histone modifications are important as histone deacetylases are targets for drugs in clinical trials for GBMs (Nagarajan and Costello 2009).

Methylation-dependent DNA sequence variation may be considered a sort of SNP (methylSNP). MethylSNPs can be easily converted into common SNPs of the C/T type by sodium bisulfite treatment of the DNA and afterwards subjected to conventional SNP typing. SnaPshot™ and Pyrosequencing™ are adapted to determine the methylation of the test CpG in a quantitative manner. The adapted methods, called SNaPmeth and PyroMeth, respectively, give nearly identical results, but data obtained with PyroMeth shows less scattering. Furthermore, the integrated software for allele frequency determination from Pyrosequencing can be used directly for data analysis while SnaPmeth data has to be exported and processed manually.

TMS1/ASC is an intracellular signaling molecule with proposed roles in the regulation of apoptosis. Whereas normal brain tissue is unmethylated at the TMS1 locus and expresses TMS1 message, human GBM cell lines exhibit reduced or absent expression of TMS1 that is associated with aberrant methylation of a CpG island in the promoter of the TMS1 gene. Progression of GBM from grade III to grade IV is associated with selective expansion of TMS1-negative cells. These findings suggest a role for the epigenetic silencing of TMS1 in the pathogenesis of

human GBM. Methylation of TMS1 may prove to be a useful prognostic biomarker and/or predictor of patient survival and tumor malignancy.

Patients with GBM have large amounts of DNA in the plasma. Of these, 90% contain methylated gene promoters, and in over 60% of these patients the same methylated promoters that are present in the tumor are also found in the plasma. This represents the first step to development of a quantitative plasma biomarker that could be used to monitor glioma status.

MCJ (DNAJD1) is a member of the DNAJ protein family whose expression is controlled epigenetically by methylation of a CpG island located within the 5′ transcribed region of its gene. Extensive methylation patterns are associated with the methylation-dependent transcriptional silencing of MCJ in medulloblastoma and further investigations of the mechanism of MCJ inactivation have revealed that its loss could occur either through biallelic epigenetic methylation or by methylation in association with genetic loss of its second allele. Epigenetic inactivation of MCJ may play a role in the development of a range of pediatric brain tumors and its role in pathogenesis and chemotherapeutic resistance needs to be investigated further.

MGMT promoter methylation has been firmly established as a biomarker in patients diagnosed with gliomas, for both clinical trials and routine clinical management (Cankovic et al. 2013). MGMT (O6-methylguanine-DNA methyltransferase) is associated with response to alkylating chemotherapy and longer survival in GBM. Specimens from patients who were treated by open resection of the tumor, followed by radiotherapy and adjuvant temozolomide chemotherapy, were investigated for MGMT promoter methylation, mRNA and protein expression, as well as presence of MGMT sequence polymorphisms (Felsberg et al. 2009). In addition, they were screened for genetic aberrations of the EGFR, TP53, CDK4, MDM2, and PDGFRA genes as well as allelic losses on chromosomal arms 1p, 10q, and 19q. Correlation of molecular findings with clinical data revealed significantly longer time to progression after onset of chemotherapy and longer overall survival of patients with MGMT-hypermethylated tumors. In contrast, MGMT protein expression, MGMT polymorphisms, and aberrations in any of the other genes and chromosomes were not significantly linked to patient outcome. Multivariate analysis identified MGMT promoter hypermethylation and near-complete tumor resection as the most important parameters associated with better prognosis. This study provides novel insights into the significance of molecular and clinical biomarkers in predicting the prognosis of GBM patients, which may improve stratification of patients into distinct prognostic subgroups.

Metabolite Biomarkers of Brain Tumors

Metabolite biomarkers of brain tumors are detected by MRS. This technique used to study a few metabolites in the brain or tumors in situ and can provide information on tumor histological type and grade as well as for monitoring treatment.

In the normal brain, the largest signals arise from N-acetylaspartate (NAA), which is confined to neurons but absent in glial cells. NAA signal is diminished or absent in brain tumors. Choline is a neurotransmitter and a component of the cell membranes. An increase in choline signal is also characteristic of brain tumors. It may indicate rapid cell division or a necrotic process associated with tumor. Lactate is an end product of anaerobic metabolism that occurs when a rapidly growing tumor does not get enough oxygen from its neovasculature. Its presence indicates cellular breakdown. Some brain tumors may show a lactate signal that is normally inverted.

In one study on patients with gliomas, 2-hydroxyglutarate (2HG) concentration as measured by MRS was reproducible and reliably reflected the disease state (Choi et al. 2016). These data provide a basis for incorporating 2HG MRS into clinical management of gliomas harboring a mutation in the gene coding for isocitrate dehydrogenase. The optimized triple refocusing MRS provides excellent 2HG signal discrimination from adjacent resonances and may confer reliable in vivo measurement of 2HG at relatively low concentrations (An et al. 2016).

miRNAs as Biomarkers of Brain Tumors

Impairment of miRNA regulatory network is one of the key mechanisms in pathogenesis of GBM. miRNA deregulation is involved in cell proliferation, apoptosis, cell cycle regulation, invasion, glioma stem cell behavior, and angiogenesis (Novakova et al. 2009). The analysis of both GBM tissues and GBM cell lines has enabled identification of a group of miRNAs whose expression is significantly altered in GBM. miR-221 is strongly up-regulated in GBM, whereas miR-128, miR-181a, miR-181b, and miR-181c, from a set of brain-enriched miRNAs, are down-regulated. In another study, plasma levels of miR-21, miR-128 and miR-342-3p were significantly altered in GBM patients compared to normal controls and could discriminate glioma from healthy controls with high specificity and sensitivity, whereas these 3 miRNAs were not significantly changed in patients with other brain tumors such as meningioma (Wang et al. 2012a). Furthermore, the plasma levels of these 3 miRNAs in GBM patients treated by operation and chemo-radiation almost dropped to normal levels. miR-128 and miR-342-3p were also positively correlated with histopathological grades of glioma.

Because the pituitary is a highly vascularized organ that releases hormones into the circulation, serum miRNAs are useful biomarkers for the diagnosis of pituitary tumors, as well as for predicting or detecting recurrence after surgery (Di Ieva et al. 2014). miR-15a and miR-16-1 genes are located at chromosome 13q14, a region which is frequently deleted in pituitary tumors. miR-15a and miR-16-1 are expressed at lower levels in pituitary adenomas as compared to normal pituitary tissue. Down-regulation of miR15 and miR16 in pituitary adenomas correlates with a greater tumor diameter and a lower p43 secretion, suggesting that these genes may, at least in part, influence tumor growth.

MRI Biomarker for Response of Brain Tumor to Therapy

Assessment of radiation and chemotherapy efficacy for brain cancer patients is traditionally accomplished by measuring changes in tumor size several months after therapy has been administered. The ability to use noninvasive imaging during the early stages of fractionated therapy to determine whether a particular treatment will be effective would provide an opportunity to optimize individual patient management and avoid unnecessary systemic toxicity, expense, and treatment delays. Pretreatment scans, and changes in tumor water diffusion values are calculated and displayed as a functional diffusion map (fDM) for correlation with clinical response. The fDMs can predict patient response at 3 weeks from the start of treatment. Early changes in tumor diffusion values could be used as a prognostic indicator of subsequent volumetric tumor response. Overall, fDM analysis provides an early biomarker for predicting treatment response in brain tumor patients.

Multigene Predictor of Outcome in GBM

No single biomarker is a predictor of outcome in GBM. An analysis using GBM microarray data from 4 independent data sets of the genes consistently associated with patient outcome revealed a consensus 38-gene survival set (Colman et al. 2010). Worse outcome was associated with increased expression of genes associated with mesenchymal differentiation and angiogenesis. Application to FFPE samples using real-time RT-PCR assays resulted in a 9-gene subset which appeared robust in these samples. This 9-gene set was then validated in an additional independent sample set. Multivariate analysis confirmed that the 9-gene set was an independent predictor of outcome after adjusting for clinical factors and methylation of the methyl-guanine methyltransferase promoter. The 9-gene profile was also positively associated with biomarkers of glioma stem-like cells, including CD133 and nestin. Finally, a multigene predictor of outcome in GBM was identified, which is applicable to routinely processed FFPE samples. The profile has potential clinical application both for optimization of therapy in GBM and for the identification of novel therapies targeting tumors refractory to standard therapy. The assay is commercially available as DecisionDx-GBM (Castle Biosciences Inc).

Neuroimaging Biomarkers Combined with DNA Microarray Analysis

Combined neuroimaging and DNA microarray analysis have been used to create a multidimensional map of gene-expression patterns in GBM that provides clinically relevant insights into tumor biology (Diehn et al. 2008). Tumor contrast enhancement and mass effect can predict activation of specific hypoxia and proliferation

gene-expression programs, respectively. Overexpression of EGFR, a receptor tyrosine kinase and potential therapeutic target, has also been directly inferred by neuroimaging and validated in an independent set of tumors by immunohistochemistry. Furthermore, imaging provides insights into the intratumoral distribution of gene-expression patterns within GBM. An "infiltrative" imaging phenotype can identify and predict patient outcome. Patients with this imaging phenotype have a greater tendency toward having multiple tumor foci and demonstrate significantly shorter survival than their counterparts. This study demonstrates a simple, widely applicable method for discovering imaging biomarkers that are associated with underlying gene-expression signatures. This approach facilitates the association of complex molecular signatures with readily identifiable imaging characteristics. These findings provide an in vivo portrait of genome-wide gene expression in GBM and offer a potential strategy for noninvasively selecting patients who may be candidates for individualized therapies.

Proteomic Analysis of CSF for Identification of Biomarkers for Gliomas

CSF is a valuable source for potential proteomic biomarkers, because changes in it can sensitively reflect CNS malignancies and 19 differentially expressed proteins have been identified (Shen et al. 2014). Further functional and protein-protein interaction assessments, performed by using Protein Analysis Through Evolutionary Relationships (PANTHER) website and Ingenuity Pathway Analysis (IPA) software, have revealed several important protein networks (e.g., IL-6/STAT-3) and four novel focus proteins (IL-6, galanin (GAL), HSPA5, and WNT4) that might be involved in glioma pathogenesis. Specifically, CSF in GBM has significantly lower GAL, HSPA5, and WNT4 levels than CSF from other grades of glioma. In contrast, IL-6 level was significantly higher in GBM when compared with other groups. Therefore, these candidate protein biomarkers, have potentials in clinical diagnosis, prognosis, treatment response monitoring, and as novel therapeutic targets in glioma.

Receptor Protein Tyrosine Phosphatase β as Biomarker of Gliomas

The receptor protein tyrosine phosphatase β (RPTPβ) is a functional biomarker for several solid tumor types. RPTPβ expression is largely restricted to the CNS and overexpressed primarily in astrocytic tumors. RPTPβ is expressed in a variety of solid tumor types with low expression in normal tissue. RPTPβ is known to facilitate tumor cell adhesion and migration through interactions with extracellular matrix components and the growth factor pleiotrophin. It is possible to generate MAbs that selectively recognize RPTPβ and kill glioma cells .

Serum Protein Fingerprinting

Screening and evaluation of protein biomarkers for the detection of GBM and their distinction from healthy individuals and benign gliomas has been done by using surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) coupled with an artificial neural network algorithm. SELDI-TOF-MS combined with bioinformatics tools, could greatly facilitate the discovery of better biomarkers. The high sensitivity and specificity achieved by the use of selected biomarkers shows great potential application for the discrimination of gliomas patients from healthy individuals and gliomas from brain benign tumors.

VEGF-R2 as Biomarker of Angiogenesis in Brain Tumors

The level of VEGF-R2 (vascular endothelial growth factor receptor 2) is elevated during angiogenesis and its levels in tumor tissues can be measured in western blots and via immunohistochemistry. A MRI molecular probe has been developed for the in vivo detection of VEGF-R2 in an experimental rodent model of glioma (Towner et al. 2010). The molecular targeting agent that was used in this study incorporated a magnetite-based dextran-coated nanoparticle backbone covalently bound to an anti-VEGF-R2 antibody. Molecular MRI with an anti-VEGF-R2 probe was shown to detect in vivo VEGF-R2 levels as a molecular biomarker for gliomas (primary brain tumors). Prussian blue staining for iron-based nanoprobes was used to confirm the specificity of the probe for VEGF-R2 in glioma tissue. Another application of this technique is in vivo assessment of angiogenesis for tissue engineering.

Future Prospects of Biomarkers of Malignant Gliomas

There has already been considerable progress in our understanding of what drives neoplastic growth in glial tumors. Further molecular characterization of these tumors in the future will accelerate biomarker discovery and facilitate the creation of new diagnostic categories for gliomas. Only isocitrate dehydrogenase mutation status (prognostic) and O6-methlyguanine methyl transferase methylation status and 1p/19q co-deletion (predictive) are currently routinely used for evaluation of glioma patients by clinicians in the US and UK. However, the ongoing development of targeted therapies as mono and combination treatments necessitates the discovery of optimally predictive molecular biomarkers, which will further our understanding of these tumors. Additionally, biomarker analysis will become a major factor in glioma clinical trials, with rapid identification of putative biomarkers in early stage trials with sufficient statistical power to validate predictive associations in phase III trials. Care will therefore be required to distinguish biomarkers that provide prognostic information from those that have predictive validity.

This approach enable future personalized therapeutic choices with minimal toxicity and improve clinical outcomes for patients in whom the diagnosis of a malignant glioma still portends a dismal outlook (Haynes et al. 2014).

Bone Tumor Biomarkers

Bone tumors are rare but have wide spectrum from benign to malignant. Bone tumors along with soft tissue cancers make up about 1% of all new cancer cases, but 10% of cancers in children and adolescents. Pathophysiology of bone tumors is still not well understood and there are technical problems of processing bone specimens for molecular studies.

Cytogenetics for the Study of Bone and Soft Tissue Tumors

Classical as well as molecular cytogenetics has been used to examine tumors for the presence of DNA abnormalities. Molecular genetic analysis of chondrosarcomas has revealed some of the abnormalities responsible for the traits of the malignant phenotype. Specific chromosomal aberrations have been described in sarcomas, which may be divided into two subgroups based on cytogenetics, one with near-diploid karyotype and few chromosomal changes, but with specific translocations and one with complex karyotypes and multiple cytogenetic aberrations. Sarcomas should be karyotyped in order to identify chromosomal changes in general, whereas FISH and PCR are the most common methods for detecting the relevant, specific translocations. Cytogenetics has been used for differentiating between benign and malignant soft tissue as well as bone tumors. For example, lipoma has 12q13, 6p and 13q changes but liposarcoma (myxoid and round cell) shows t(12;16)(q13;p11) rearrangement.

Biomarkers of Ewing's Tumors

Ewing sarcoma family tumors (ESFTs), which affect both bone and soft tissues, includes Ewing sarcoma and primitive neuroectodermal tumor. ESFTs are aggressive tumors of putative stem cell origin for which prognostic biomarkers and novel treatments are needed. Histologically, these tumors are composed of sheets of small round cells with minimal cytoplasm. Immunohistochemical biomarkers greatly facilitate differentiating these lesions from mimics. In particular, CD99 overexpression can be detected in most tumors. Early cytogenetic analysis points to a characteristic t(11;22) translocation. Molecular methods used to facilitate the diagnosis of Ewing sarcoma (EWS) generally include FISH and RT-PCR. FISH for EWS using the break-apart technique has the advantage of detecting a broad array of translocations, making this a useful screening test; a small number of translocation negative

cases may be resolved by also assessing for the translocation of fusion genes. Proteomic studies have shown that nucleophosmin is a prognostic biomarker of EWS that correlates with survival (Kikuta et al. 2009).

BMI-1 polycomb protein is overexpressed by the vast majority of ESFTs and is associated with poor prognosis. However, in 20% of cases, BMI-1 levels are low or undetectable. Although clinical presentation and outcome are similar between BMI-1-high and BMI-1-low tumors, whole genome expression array analysis shows marked differences in their respective gene expression profiles. Gene-specific enrichment analysis has identified several cancer-associated canonical biological pathways, including IGF1, mTOR, and WNT, which are significantly downregulated in BMI-1-low compared to BMI-1-high tumors (Cooper et al. 2011). Consistent with these in vivo data, the response to IGF1-R inhibition in vitro is diminished in BMI-1-low compared with BMI-1-high ESFT cells.

Role of Biomarkers in the Diagnosis of Bone Tumors

If molecular biomarkers are used in the diagnosis of bone tumors (e.g. Ewing sarcoma), they should not form the basis of the diagnosis by themselves; this information should be integrated with the clinical, radiologic and pathologic features of the tumor. Ultrastructural, immunohistochemical, cytogenetic and molecular studies should be used to supplement histological observations. Whole genome sequencing from formalin-fixed, paraffin-embedded tissue promises to provide a wealth of information regarding bone tumor genetics (Dickson and Kandel 2010).

Breast Cancer Biomarkers

Breast cancer is one of the most common diseases affecting women. More than 250,000 women are diagnosed with breast cancer every year in the US. According to the National Cancer Institute, ~13% of women in the US will develop breast cancer during their lifetime. The cause of breast cancer is multifactorial, involving environmental, hormonal, and genetic factors. Early detection of breast cancer is important for management and prognosis. Mammography and ultrasound are the most commonly used among the various methods used currently for screening for breast cancer. Various biomarkers for cancer are shown in Table 13.7. Important biomarkers of breast cancer include genes and HER-2/neu oncoprotein.

Autoantibody Biomarkers of Breast Cancer

Biomarkers are potentially useful for diagnosis of basal breast tumors, which are particularly difficult to diagnose with mammograms. Similar to these cancers, ER-negative breast cancers are detected through other methods as they often occur

Table 13.7 Biomarkers of breast cancer

Detection of predisposition to breast cancer
Cancer gene profiling: BRCA1, BRCA2 and EMSY
Proneurotensin (pro-NT) and Proenkephalin (pro-ENK)
Proteomic biomarkers for early detection of breast cancer
Autoantibody biomarkers of breast cancer
Cdk6
Epithelial growth factor receptor (EGFR) levels in blood elevated due to mutations of EGFR gene
High mobility group protein A2
Mammaglobin
Progranulin
Riboflavin carrier protein
Serum proteomic profiling
Suppressor of deltex protein
Breath biomarkers of breast cancer: mixture of organic volatile compounds
Antigens as biomarkers of breast cancer
Serum CEA: prognostic factors for locally advanced breast cancer
Proliferating cell nuclear antigen: isoform associated with malignancy
miRNA biomarkers of breast cancer
Biomarkers for identifying patients at high risk for distant metastases
Cyclin E
CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule 6)
Dachshund gene (DACH1)
Gene ratio: homeobox 13 (HOXB13) and interleukin-17B receptor (IL17BR)
Hypermethylation biomarkers: 14-3-3-δ gene
Podocalyxin: a CD34-related transmembrane protein
Protein kinase C epsilon
UPA/PAI-1
Predictive biomarkers for response to therapeutics agents
Prediction of response to endocrine therapy: estrogen receptor/progesterone receptor
Predictors of response to anti-estrogen therapy: retinoblastoma tumor suppressor gene
Predictors of response to cytotoxic chemotherapy
Predictors of response to chorionic gonadotrophin
Predictors of response to tamoxifen (decrease of level of insulin-like growth factor)
Predictors of response to trastuzumab (Herceptin): HER 2 gene overexpression
Biomarkers of prognosis of breast cancer
Carbonic anhydrase IX (CA IX) indicates poor prognosis in postmenopausal women with breast cancer
Centromere protein-F (CENP-F) is a gene associated with poor prognosis in breast cancer
Cytokeratins
GP88: elevation in women with ER+ breast cancer is associated with 4-fold increased risk of progression
High expressing levels of p16 and/or COX-2, when coupled with tumor proliferation (low or high)
Lipocalin 2 (Lcn2) promotes breast cancer progression
p27 expression as biomarker for survival after treatment with adjuvant chemotherapy
Serum CA 15-3 and CA 27-29: prognostic factors for locally advanced breast cancer
Type III TGF-β receptor as regulator of cancer progression

in younger women, who tend to have denser breast tissue, which makes mammography less successful. These women could also benefit from an additional blood test to pick up biomarkers. Autoantibodies are promising blood-based biomarkers. Although individual autoantibodies are unlikely to enhance early detection, multiplexed assays for autoantibody panels may achieve the required sensitivity. Nucleic Acid Programmable Protein Arrays (NAPPA) for protein-protein interactions are high-density, protein arrays that could be customized for this purpose (Tang et al. 2017). The idea is to display several different proteins in a sample so the autoantibodies in a patient's serum can find any proteins they happen to recognize. The arrays need to be fairly stable and should display proteins that will not change or unfold over time. The NAPPA approach is to take cloned copies of genes and print them on the array. The cloned copies, cDNA, and are configured in such a way that an epitope tag can be added at the C-terminus of the gene. Anything that is captured by virtue of the tag must have the full-length protein attached to it. Those genes are printed on the array, which can then be stored for months. Once the array is needed, it is floated in expression extract that transcribes and translates the proteins in situ on the glass. That proteins are made about an hour before they are used, guaranteeing that they are as fresh as possible.

The technology uses minimal samples, as little as 0.01 ml of plasma or serum, and has good assay reproducibility and reliability based on pilot work, making it particularly attractive for molecular epidemiology studies. NAPPA technology and other proteomic platforms are another extension of genomic technologies that might provide novel biomarkers for breast cancer. However, this is a new area and the biology of autoantibodies is not well understood. There is need to learn how age and lifestyle factors, as well as intra-person and inter-person variability, influence these biomarkers. Because basal breast cancers are highly aggressive, it may not be possible to isolate autoantibody biomarkers appropriate for early detection, and one may only find biomarkers related to overall tumor aggressiveness.

The ultimate goal of the research is to identify candidate autoantibody biomarkers that may detect breast cancers early or predict survival. Athough several studies have investigated the diagnostic use of tumor-associated autoantibodies as biomarkers for detection of breast cancer, a high-quality prospective study is needed to validate their diagnostic value in practice (Xia et al. 2016).

Biomarkers of Breast Cancer in Breath

A breath test for volatile organic compounds (VOCs) as a predictor of breast cancer has been developed by Menssana Research Inc. Breath VOCs, apparently resulting from increased oxidative stress and cytochrome p450 induction, are assayed in asymptomatic women with abnormal mammograms and biopsy-proven breast cancer, and compared to control subjects of age-matched healthy women. A mobile point-of-care system collects and concentrated breath and air VOCs for analysis with gas chromatography and surface acoustic wave detection. Chromatograms are segmented into a time series of alveolar gradients (breath minus room air). Segmental

alveolar gradients are ranked as candidate biomarkers by C-statistic value. Multivariate predictive algorithms are constructed employing significant biomarkers identified with multiple Monte Carlo simulations and cross validated with a leave-one-out procedure. A pilot study of a 6-minute point-of-care breath test for volatile biomarkers accurately identified women with breast cancer and with abnormal mammograms (Phillips et al. 2014). Breath testing could potentially reduce the number of needless mammograms without loss of diagnostic sensitivity.

Biomarkers for Breast Cancer in Nipple Aspiration Fluid

Breast fluid is a rich source of breast cancer biomarkers. Ductal lavage is a method of minimal epithelial sampling of the breast, with potential utility for repeat sampling and biomarker analysis. In combination with high-throughput novel proteomic profiling technology and multicenter study design, biomarkers that are highly specific to breast cancer can be discovered and validated. Persistent elevation of human neutrophil peptide in high-risk women may imply early onset of cancer not yet detectable by current detection method. Proof of this hypothesis requires studies on larger study populations.

Circulating Tumor DNA as Biomarker of Breast Cancer

Monitoring of response to treatment of breast cancer is essential for avoiding continual of ineffective therapies to prevent unnecessary side effects, and to determine the benefit of new therapies. Treatment response is generally assessed with the use of serial imaging, but radiographic measurements often fail to detect changes in tumor burden. Serum biomarker CA 15-3 is clinically useful in some patients with metastatic breast cancer but it has a sensitivity of only 60–70%. Therefore, there is an urgent need for biomarkers that measure tumor burden with high sensitivity and specificity.

Circulating nucleic acids (CNA) isolated from serum or plasma are increasingly recognized as biomarkers for cancers. NGS provides high numbers of DNA sequences to detect the trace amounts of unique serum biomarkers associated with breast carcinoma. Serum CNA of women with ductal carcinoma was extracted and sequenced on a 454/Roche high-throughput GS-FLX platform and compared with healthy controls and patients with other medical conditions (Beck et al. 2010). Breast cancer was accurately detected at a diagnostic specificity level of 95% with a calculated sensitivity of 90%. Identification of specific breast cancer-related CNA sequences provides the basis for the development of a serum-based routine laboratory test for breast cancer screening and monitoring. This is in development by Chronix Biomedical.

For the detection of metastatic breast cancer, circulating tumor DNA shows superior sensitivity to that of other circulating biomarkers and has a greater dynamic range that correlates with changes in tumor burden (Dawson et al. 2013). Circulating tumor DNA often provides the earliest measure of treatment response.

Circulating Exosomes as Biomarkers of Breast Cancer

The proportion of clinically relevant exosomes (CREs) in circulating blood can enable staging of cancer as well as non-invasive monitoring of interindividual variations in tumor-receptor expression levels. One approach to quantification of CREs utilizes a Surface Plasmon Resonance (SPR) platform to determine the proportion of CREs in a two-step strategy that involves; (i) initial isolation of bulk exosome population using tetraspanin biomarkers (i.e., CD9, CD63); and (ii) subsequent detection of CREs within the captured bulk exosomes using tumor-specific biomarkers such as HER2 (Sina et al. 2016). The authors demonstrated isolation of bulk exosome population and detection of as low as 10% HER2-positive exosomes from samples containing designated proportions of HER2-positive BT474 and HER2-negative MDA-MB-231 cell-derived exosomes. Exosomes were successful isolated from a small cohort of breast cancer patient samples with identification of ~14–35% of their bulk population expressing HER2.

Flow Cytometry for Quantification of Biomarker Expression Patterns

The established method in prognosis of breast cancer includes detection of molecular markers, such as the estrogen receptor (ER), progesterone receptor (PR), and HER-2/neu. These markers are routinely checked via immunohistochemistry (IHC). HER-2/neu is also detected by fluorescent in situ hybridization (FISH). Flow cytometric analysis can provides quantitative data on expression patterns of important prognostic markers in breast cancer and has been used for the detection of ER, PR, HER-2/neu, epidermal growth factor receptor (EGFR), and E-cadherin. Currently, EGFR and E-cadherin are not standard predictive factors in determining survival of breast cancer patients, but both may be beneficial for determining prognosis in the future. Cells undergoing flow cytometric analysis lose marker expression with increasing number of passages. The highest expression is found at cells passaged 0–1 times. ER, PR, and HER-2/neu marker expressions in 5 out of 5 cell lines were consistent with established expression patterns. EGFR and E-cadherin expression in 4 out of 5 cell lines are also consistent with established expression patterns.

Plasma Proteomics for Biomarkers of Breast Cancer

Protein-based breast cancer biomarkers are a promising resource for breast cancer detection at the earliest and most treatable stages of the disease. Plasma is well suited to proteomic-based methods of biomarker discovery because it is easily obtained, is routinely used in the diagnosis of many diseases, and has a rich proteome. However, due to the vast dynamic range in protein concentration and the often uncertain tissue and cellular origin of plasma proteins, proteomic analysis of plasma requires special consideration compared with tissue and cultured cells. For example, when studies report an upregulation of IL-6 in the serum of breast cancer patients compared with control individuals, it is difficult to know whether this

protein is released directly from the tumor or whether IL-6 upregulation is a systemic reaction to the tumor and released by nontumor tissues.

Biomarkers should be tissue specific in addition to being tumor specific. If cancer is detected, but not the tissue of origin, it may create problems, since searching for a suspected tumor will add undo stress to the patient and increased cost to the treatment. Finding tissue-specific tumor markers has thus far proven difficult. Many candidate biomarkers have been concurrently identified in numerous tumor types. This likely reflects that fact that 90% of all cancers are of epithelial origin and thus express many of the same proteins. It is probable that a panel of biomarkers will be required to establish tissue specificity rather than a single protein; this panel may or may not be independent of a tumor-specific panel of biomarkers. In addition, early detection biomarkers may need to be used in conjunction with other screening methods, such as mammography, where the tissue of origin is not in question.

Quantitative Realtime PCR Assays for Biomarker Validation

Microarray analysis and a QRT-PCR assay can be used to risk-stratify breast cancers based on biological 'intrinsic' subtypes and proliferation. QRT-PCR is attractive for clinical use because it is fast, reproducible, tissue-sparing, quantitative, automatable, and can be performed from archived (formalin-fixed, paraffin-embedded tissue) samples. The benefit of using QRT-PCR for cancer diagnostics is that new biomarkers can be readily validated and implemented, making tests expandable and/or tailored to the individual. For instance, the proliferation metagene could be used within the context of the intrinsic subtypes or used as an ancillary test in breast cancer and other tumor types where an objective and quantitative measure of grade is important for risk stratification. As more prognostic and predictive signatures are discovered from microarray, it should be possible to build on the current biological classification and develop customized assays for each tumor subtype. This approach enables the important clinical distinction between ER-positive and ER-negative tumors and identifies additional subtypes that have prognostic value. The proliferation metagene offers an objective and quantitative measurement for grade and adds significant prognostic information to the biological subtypes. It is a robust predictor of survival across all breast cancer patients and is particularly important for prognosis in Luminal A (ER-positive) breast cancers, which have a worse outcome than expected when proliferation is high. This supports previous findings that a genomic signature of proliferation is important for predicting relapse in breast cancer, especially in ER-positive patients.

The quantitative mRNA expression levels of ERa, PgR and HER2 strongly correlate with the respective quantitative protein expression levels prospectively detected by EIA. In addition, HER2 mRNA expression levels correlated well with gene amplification detected by FISH in the same biopsies. These results indicate that both QRT-PCR methods were robust and sensitive tools for routine diagnostics and consistent with standard methods. The simultaneous assessment of several biomarkers is fast as well as labor effective and optimizes clinical decision-making process in breast cancer tissue and/or core biopsies.

Cdk6 as a Biomarker of Breast Cancer

Normal human mammary epithelial cells have a high amount of cyclin-dependent kinase-6 (cdk6) protein and activity, but all breast tumor-derived cell lines analyzed have reduced levels, with several having little or no cdk6 and these can be restored to those characteristic of normal human mammary epithelial cells by DNA transfection. cdk6 may be useful as a cancer biomarker and as a target for cancer therapy in patients with breast cancer. Potential applications are:

- Diagnostic assay for breast cancer and for determining the stage of malignancy.
- Predictor of in vivo tumor cell growth.
- Diagnostic assays for evaluating the efficacy of anticancer treatments.
- Method to regulate tumor cell growth.

Centromere Protein-F

Reanalysis of a high profile breast cancer DNA microarray dataset containing 96 breast tumor samples and use of a powerful statistical approach, between group analyses, led to the identification of centromere protein-F (CENP-F), a gene associated with poor prognosis (O'Brien et al. 2007). In a published follow-up breast cancer DNA microarray study, comprising 295 tumour samples, CENP-F upregulation was found to be significantly associated with worse overall survival and reduced metastasis-free survival. To validate and expand upon these findings, the authors used two independent breast cancer patient cohorts represented on tissue microarrays. CENP-F protein expression was evaluated by immunohistochemistry in 91 primary breast cancer samples from cohort I and 289 samples from cohort II. CENP-F correlated with markers of aggressive tumor behavior including ER negativity and high tumor grade. In cohort I, CENP-F was significantly associated with markers of CIN including cyclin E, increased telomerase activity, c-Myc amplification and aneuploidy. In cohort II, CENP-F correlated with VEGFR2, phosphorylated Ets-2 and Ki67, and in multivariate analysis, was an independent predictor of worse breast cancer-specific survival and overall survival. In conclusion, CENP-F is associated with poor outcome in breast cancer.

Carbonic Anhydrase IX

Hypoxia in breast cancer is associated with poor prognosis and down-regulation of the estrogen receptor (ER). Carbonic anhydrase IX (CA IX) is a hypoxia-inducible gene that has been associated with poor outcome in many epithelial cancers. CA IX expression is associated with a reduced relapse-free survival, overall survival, and breast cancer-specific survival. Multivariate analysis shows that CA IX is an independent prognostic biomarker in untreated patients with one to three positive lymph nodes. Thus, CA IX is biomarker of poor prognosis in premenopausal breast cancer patients and may be associated with resistance to radiotherapy.

COX-2 as a Biomarker of Breast Cancer

Cyxlooxygenase (COX) enzymes produce prostaglandin compounds responsible for pain and inflammation, and nonsteroidal anti-inflammatory drugs (NSAIDs) are designed to reduce expression of COX enzymes, although some NSAID use has been associated with side effects (most notably possible kidney failure). COX-2 is a form of COX that is not usually found in normal tissues but which has been associated with several cancers, including ductal carcinoma in situ and invasive breast cancers.

Atypical hyperplasia in breast tissue, although benign, is associated with a high risk of breast cancer. A study has assessed the relationship between risk of breast cancer and COX-2 expression in archival specimens from women with atypical hyperplasia and a 15-year follow-up (Visscher et al. 2008). The risk for developing breast cancer increased with increasing COX-2 expression. Overexpression of COX-2 was statistically significantly associated with the type of atypia, with number of foci of atypia in the biopsy, and with older age at time of biopsy. Specifically, 20 years after a biopsy in which atypia was found, 31% of women with high levels of COX-2 in their atypia sample had developed breast cancer, versus 14% of those with no COX-2 expression. For those with moderate levels of COX-2, 24% had developed breast cancer. This study indicates that COX-2 may be a biomarker that further stratifies breast cancer risk among women with atypia and may be a relevant target for chemoprevention strategies, e.g. COX-2 inhibitors such as celecoxib or rofecoxib.

G88 as a Biomarker of Progression of ER+ Breast Cancer

Out of 250,000 cases of breast cancer per year in the US, ~175,000 are estrogen receptor (ER) positive and receive a treatment regimen that includes anti-estrogen compounds such as tamoxifen. Increased level of a protein biomarker GP88 (progranulin) in these tumors is an indicator of 4-fold increased risk of disease progression. Further clinical studies indicate that GP88 levels are elevated in breast cancer patients as compared to normal controls. A&G Pharmaceutical is developing a MAb against GP88 as personalized therapy.

Glycomic Biomarkers of Breast Cancer

Since the glycosylation of proteins is known to change in tumor cells during the development of breast cancer, a glycomic approach has been investigated to find relevant biomarkers of breast cancer. These glycosylation changes are known to correlate with increasing tumor burden and poor prognosis. Current antibody-based immunochemical tests for cancer biomarkers of ovarian (CA125 or MUC16), breast (CA 27.29 or CA15-3), pancreatic, gastric, colonic and ovarian carcinoma (CA19-9), target highly glycosylated mucin proteins. However, these tests lack the

specificity and sensitivity for use in early detection. The glycomics approach to find glycan biomarkers of breast cancer involves chemically cleaving glycans from glycosylated proteins that are shed or secreted by breast cancer tumor cell lines (Kirmiz et al. 2007). The resulting free glycan species are analyzed by MALDI Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR MS). Further structural analysis of the glycans can be performed in FTMS through the use of tandem mass spectrometry with infrared multi-photon dissociation. These methods were then used to analyze sera obtained from a mouse model of breast cancer, and a small number of serum samples obtained from human patients diagnosed with breast cancer, or patients with no known history of breast cancer. In addition to the glycosylation changes detected in mice as mouse mammary tumors developed, glycosylation profiles were found to be sufficiently different so as to distinguish patients with cancer from those without. Although the small number of patient samples analyzed so far is inadequate to make any legitimate claims at this time, these promising, but very preliminary results suggest that glycan profiles may contain distinct glycan biomarkers that may correspond to glycan "signatures of cancer".

HER-2/neu Oncoprotein

Overexpression of members of the human epidermal growth factor receptor (HER) family has been widely studied in breast cancer. HER-2/neu oncoprotein has been widely studied for many years and has been shown to play a pivotal role in the development and progression of breast cancer. HER2/neu has been shown to be an indicator of poor prognosis with patients exhibiting aggressive disease, decreased overall survival and a higher probability of recurrence of disease. Elevated levels of HER2/neu are found not only in breast cancer but also in several other tumor types including prostate, lung, pancreatic, colon and ovarian cancers. As evidenced by numerous published studies, elevated levels of HER2/neu (also referred to as overexpression) are found in about 30% of women with breast cancer. Determination of a patient's HER2/neu status may be valuable in identifying whether that patient has a more aggressive disease and would, thus, derive substantial benefit from more intensive or alternative therapy regimens. Some studies suggest that in certain breast cancer patients, persistently rising HER2/neu values may be associated with aggressive cancer and poor response to therapy, while decreasing HER2/neu levels may be indicative of effective therapy. Randomized, controlled clinical trials have shown that amplification of HER2 in breast-cancer cells is associated with better clinical responsiveness to anthracycline-containing chemotherapy regimen when compared with the regimen of cyclophosphamide, methotrexate, and fluorouracil.

Traditional HER2/neu testing is generally limited to tissue from primary breast cancer and does not provide information regarding the HER2/neu status in women with recurrent, metastatic breast cancer. The introduction of microtiter plate ELISA HER-2/neu testing using a serum sample now offers a less invasive diagnostic tool and provides a current assessment of a woman's HER2/neu status over the course of disease. Immunohistochemistry (IHC) analysis of HER2/neu in breast carcinoma is

a useful predictor of response to therapy with trastuzumab when strongly positive. Negative immunostaining is highly concordant with a lack of gene amplification by FISH. Most weakly positive overexpressors are false-positives on testing with FISH. Thus, screening of breast carcinomas with IHC and confirmation of weakly positive IHC results by FISH is an effective evolving strategy for testing HER2/neu as a predictor of response to targeted therapy. The clinical utility of the serum test as a prognostic indicator has not yet been fully established but is under investigation. Findings from a study using serum HER2/neu test showed that proven metastatic breast cancer patients whose serum HER2/neu levels decreased by less than 20% experienced decreased benefit from trastuzumab-based therapy.

Potential mechanisms of trastuzumab resistance include altered receptor-antibody interaction, increased cell signaling from other HER receptors, increased Akt activity, reduced PTEN level, reduced p27kip1, and increased IGF-IR signaling. There is an urgent need to identify biomarkers to guide anti-HER2 therapy in patients who develop progressive metastatic breast cancer (MBC) while receiving trastuzumab, and to identify combination therapies using novel anti-HER2 agents. In a pooled analysis of patients with MBC, individuals who did not achieve a significant decline in serum HER-2/neu levels had decreased benefit from trastuzumab-based therapy, and these patients should be considered for clinical trials evaluating additional HER-2/neu-targeted interventions (Ali et al. 2008).

Current methods for checking HER2 are problematic because of issues with intra- and inter-laboratory reproducibility and preanalytic variables, such as fixation time. In addition, the commonly used HER2/chromosome 17 ratio presumes that chromosome 17 polysomy is present when the centromere is amplified, even though analysis of the rest of the chromosome is not included in the assay. In one study, 97 frozen samples of invasive lobular and invasive ductal carcinoma, with known ICH and FISH results for HER2, were analyzed by aCGH to a commercially available bacterial artificial chromosome whole-genome array containing 99 probes targeted to chromosome 17 and the HER2/TOP2 amplicon (Yeh et al. 2009). Results were 97% concordant for HER2 status, meeting the College of American Pathologists/American Society of Clinical Oncology's validation requirements for HER2 testing. No case of complete polysomy 17 was detected even though multiple breast cancer cases showed polysomies of other chromosomes. Therefore, aCGH is an accurate and objective DNA-based alternative for clinical evaluation of HER2 gene copy number, and that polysomy 17 is a rare event in breast cancer.

High Mobility Group Protein A2

High mobility group protein A2 (HMGA2) is a transcription factors that is expressed during embryonic development, but not in normal adult tissues. The HMGI family consists of three proteins, HMGI, HMGI(Y) and HMGA2 (also known as HMGI-C). Experiments with knockout HMGA2 −/− mice yielded a reduced body weight compared to wild-type HMGA2 +/+ mice, which indicates that HMGA2 plays a

role in mammalian growth. Interest in HMGA2 has increased recently as it has been found that HMGA2 is expressed in neoplastic tissues and that it apparently has a role in control of cell growth, differentiation and tumorigenesis. HMGA2 is expressed in tumor tissue but not in normal tissue immediately adjacent to the tumor tissue. HMGA2 initiates sustained silencing of E-cadherin expression via the epithelial-mesenchymal transition transcription factors, thus achieving and promoting tumor cell invasion (Tan et al. 2015). Studies in peripheral blood show that HMGA2 is not present in normal healthy donors, but is present in the blood of a subset of breast cancer patients. In general, the presence of HMGA2 in the peripheral blood of breast cancer patients has a correlation with poor survival and with a higher histologic grade of the tumor. It is a potential biomarkers of breast cancer.

Hypermethylated Genes as Biomarkers of Metastatic Breast Cancer

Numerous hypermethylated genes have been reported in breast cancer, and the silencing of these genes plays an important role in carcinogenesis, tumor progression and diagnosis. These hypermethylated promoters are very rarely found in normal breast. Aberrant hypermethylation may be useful as a biomarker, with implications for breast cancer etiology, diagnosis, and management.

A study found that serum levels of methylated gene promoter 14-3-3-δ (Stratifin) significantly differed between control and metastatic breast cancer groups, and between disease-free and metastatic breast cancer groups (Zurita et al. 2010). The ratio of the 14-3-3- δ level before the first chemotherapy cycle to the level just before administration of the second chemotherapy cycle, defined as the Biomarker Response Ratio (BRR), was calculated for the "continuous decline" and "rise-and-fall" groups. Subsequent ROC analysis showed a sensitivity of 75% and a specificity of 66.7% for discriminating between the groups for a cut-off level of BRR = 2.39. The relationship of 14-3-3-sigma with breast cancer metastasis and progression found in this study suggests a possible application of 14-3-3-sigma as a biomarker to screen for metastasis and for follow-up of patients treated for metastatic breast cancer by monitoring their disease status and treatment response.

Lipocalin 2 as Biomarker of Breast Cancer Progression

Lipocalin 2 (Lcn2) promotes breast cancer progression, and the mechanisms underlying this function have been identified (Yang et al. 2009). Lcn2 levels are consistently associated with invasive breast cancer in human tissue and urine samples. Lcn2 is overexpressed in human breast cancer cells and upregulates mesenchymal markers, including vimentin and fibronectin, downregulates the epithelial marker E-cadherin, and significantly increase cell motility and invasiveness. These changes in marker expression and cell motility are hallmarks of an epithelial to mesenchymal transition (EMT). In contrast, Lcn2 silencing in aggressive breast cancer cells

inhibits cell migration and the mesenchymal phenotype. Furthermore, reduced expression of estrogen receptor (ER) alpha and increased expression of the key EMT transcription factor Slug are observed with Lcn2 expression. Overexpression of ERalpha in Lcn2-expressing cells reverses EMT and reduces Slug expression, suggesting that ERalpha negatively regulates Lcn2-induced EMT. Finally, Lcn2-expressing breast tumors display a poorly differentiated phenotype and show increased local tumor invasion and lymph node metastasis. Taken together, these in vitro, in vivo, and human studies demonstrate that Lcn2 promotes breast cancer progression by inducing EMT through the ERalpha/Slug axis and may be a useful biomarker of breast cancer.

Long Intervening non-coding RNAs

Long intervening non-coding RNAs (lincRNAs) in the HOX loci become systematically dysregulated during breast cancer progression. The lincRNA termed HOTAIR is increased in expression in primary breast tumors and metastases, and HOTAIR expression level in primary tumors is a powerful predictor of eventual metastasis and death (Gupta et al. 2010). Enforced expression of HOTAIR in epithelial cancer cells induced genome-wide re-targeting of Polycomb repressive complex 2 (PRC2) to an occupancy pattern more resembling embryonic fibroblasts, leading to altered histone H3 lysine 27 methylation, gene expression, and increased cancer invasiveness and metastasis in a manner dependent on PRC2. Conversely, loss of HOTAIR can inhibit cancer invasiveness, particularly in cells that possess excessive PRC2 activity. TaqMan non-coding RNA assays (ThermoFisher Scientific) can accurately measure expression levels of this molecular biomarker in different breast cancer samples and have helped to uncover regulatory roles of non-coding RNAs in breast cancer.

Mammaglobin

Mammaglobin, a glycoprotein, is almost exclusively expressed in breast epithelial cells. It is frequently elevated in breast cancer. An ELISA can detect mammaglobin protein in serum by immunostaining and is highly sensitive and specific for detection of mammaglobin protein in tissue culture fluids of breast cancer cells and sera of breast cancer patients. ELISA differentiates healthy women from those with breast cancer with accurate, repeatable results across time and under varying storage conditions indicating that mammaglobin, as measured by the ELISA, holds significant promise for breast cancer screening with the realistic potential to impact management of this disease. Mammaglobin is being explored as a target for immune-based interventions. In vitro studies have demonstrated that T cell-mediated immune responses can be induced against mammaglobin-derived peptides expressed by MHC molecules on tumor cells and antigen-presenting cells.

miRNA Biomarkers of Breast Cancer

Altered abundance of cell cycle regulation proteins and aberrant expression of miRNAs frequently coexist in human breast cancers. Altered miRNA expression in breast cancer cell lines is associated with altered cell cycle progression and cell proliferation. Recent studies have demonstrated a causal role for miRNA in governing breast tumor suppression or collaborative oncogenesis (Yu et al. 2010). Various miRNAs associated with breast cancer are shown in Table 13.8.

Global testing pathway analysis showed an association of hsa-miRNA-30c expression with HER and RAC1 signaling pathways (Rodríguez-González et al. 2011). The authors identified hsa-miRNA-30c as an independent predictor for clinical benefit of tamoxifen therapy in patients with advanced breast cancer. Assessment of tumor levels and connected pathways could be helpful to improve treatment strategies.

Table 13.8 miRNA associated with breast cancer

miRNA	Expression status in fresh frozen breast cancer tissue specimens
miR-7	Expression associated with tumor aggressiveness
miR-10b	Downregulated in tumors in patients with distant and regional relapse and local recurrence
miR-21	Overexpression correlated with advanced clinical stage, lymph node metastasis and poor prognosis
miR-125a	Downregulated in tumors
miR-125b	Downregulated in tumors
miR-128	Expression associated with tumor aggressiveness
miR-145	Downregulated in tumors
miR-155	Upregulated in tumors
miR-205	Downregulated in tumors
miR-206	Downregulated in ER + tumors
miR-210	Expression associated with tumor aggressiveness, early relapse and poor outcome
miR-93	Highly expressed in high-grade tumors
miR-106b	Highly expressed in high-grade tumors
miR-25	Highly expressed in high-grade tumors
miR-335	Increased expression led to metastasis suppression
miR-126	Increased expression led to metastasis suppression
miR-373	Increased expression stimulated cell migration and invasion
miR-516-3p	Expression associated with tumor aggressiveness
miR-520c	Increased expression stimulated cell migration and invasion
miR-27b	Downregulated in tumors
miR-17-5p	Downregulated in tumors
miR-9-1	Downregulated in tumors

p27 Expression as Biomarker for Survival after Chemotherapy

Abnormal expression of the cell cycle regulatory proteins p27(Kip1) may be associated with breast cancer survival and relapse. Low p27 expression is associated with poor prognosis, especially among patients with steroid receptor-positive tumors. Specifically, the 5-year survival is >91% in women whose tumors have high p27 expression, as compared to a survival rate of <85% in women whose tumors exhibit low p27 expression. There is no association between p27 expression and decreased survival among women with hormone-receptor-negative tumors. p27 may be a useful biomarker for predicting breast-cancer mortality, but more work needs to be done before widespread use. Currently, it appears to be most useful for predicting outcome and tailoring treatment in women whose tumors are hormone-receptor positive.

Podocalyxin

Podocalyxin is a CD34-related transmembrane protein involved in hematopoietic cell homing and breast cancer progression but the mechanisms involved are not clear. It has, however, been postulated that the adaptor proteins NHERF-1 and 2 could regulate apical targeting of podocalyxin by linking it to the actin cytoskeleton. However, a new study has found that full-length podocalyxin acts to recruit NHERF-1 to the apical domain (Nielsen et al. 2007). Podocalyxin was found to significantly expands the non-adhesive face of cells, allowing individual cells to brush aside adhesion molecules situated between tumour cells. The freed cells then move away from the original site to form new tumors at other sites. Also, the protein causes tumor cells to sprout microvilli, or hair-like projections, that may help propel cancer cells to metastasize to other sites. The discovery demonstrated that the protein not only predicted the spread of breast cancer cells, it likely helped to cause it. The data from this study suggest that this single molecule can modulate NHERF localization and, independently, act as a key orchestrator of apical cell morphology, thereby lending mechanistic insights into its multiple roles as a polarity regulator, tumor progression marker, and anti-adhesin. The mechanism is now believed to apply to difficult-to-treat invasive breast and ovarian cancers. Next steps involve advancing the research in animal models, designing antibodies to block the function of the protein and identifying new therapies to combat metastasizing cancer.

Proneurotensin and Proenkephalin

Proneurotensin (pro-NT) und Proenkephalin (pro-ENK) are biomarkers of breast cancer risk prediction. Fasting pro-NT is significantly associated with the development of diabetes, cardiovascular disease, breast cancer, and with total as well as cardiovascular mortality (Melander et al. 2012). Women with high pro-NT have 8-fold and those with low levels of pro-NK have 20-fold increased risk of developing breast cancer.

Neurotensin (NT) is released in the gastrointestinal tract and the major stimulus for its release is fat uptake. NT plays a role as satiety hormone. The peptide is not stable in blood and, therefore, not suitable for routine diagnosis. Sphingotec discoverd pro-NT as a stable and useful surrogate biomarker for NT. Sphingotest® pro-NT is an assay for in vitro determination Pro-NT.

Proliferating Cell Nuclear Antigen

Two isoforms of proliferating cell nuclear antigen (PCNA) have been observed in breast cancer cells. Commercially available antibodies to PCNA recognize both isoforms and, therefore, cannot differentiate between the PCNA isoforms in malignant and nonmalignant breast epithelial cells and tissues. A unique antibody has been developed that specifically detects a PCNA isoform (caPCNA) associated with breast cancer epithelial cells grown in culture and breast-tumor tissues. Immunostaining studies using this antibody suggest that the caPCNA isoform may be useful as a biomarker of breast cancer and that the caPCNA-specific antibody could potentially serve as a highly effective detector of malignancy. It was also shown that the caPCNA isoform functions in breast cancer-cell DNA replication and interacts with DNA polymerase delta. These studies indicate that the caPCNA isoform may be a previously uncharacterized biomarker of breast cancer.

Protein Kinase C as a Predictive Biomarker of Metastatic Breast Cancer

Protein kinase C epsilon (PKC epsilon), a member of a family of serine/threonine protein kinases, is a transforming oncogene that has been reported to be involved in cell invasion and motility. High-density tissue microarray analysis shows that PKC epsilon plays a role in breast cancer development and progression. Increasing PKC epsilon staining intensity is associated with high histologic grade, positive Her2/neu receptor status, and negative estrogen and progesterone receptor status. PKC epsilon is a validated target for RNA interference (RNAi) anticancer therapy based on the demonstration of in vivo inhibition of metastases in animals with experimental tumors. These findings show that PKC epsilon plays a critical and causative role in promoting an aggressive metastatic breast cancer phenotype and as a target for anticancer therapy.

Retinoblastoma Tumor Suppressor Gene as a Biomarker

According to the NCI, about two-thirds of women with breast cancer have estrogen-receptor-positive breast cancer, in which tumor growth is regulated by the natural female hormone estrogen. Estrogen is known to promote the growth of most types of breast cancer. However, another gene, the retinoblastoma tumor suppressor (RB) gene, is functionally inactivated in the majority of human cancers and is aberrant in one-third of all breast cancers. RB regulates G1/S-phase cell-cycle progression and

is a critical mediator of antiproliferative signaling. RB deficiency compromises the short-term cell-cycle inhibition following cisplatin, ionizing radiation, and anti-estrogen therapy of breast cancer with drugs such as tamoxifen (Bosco et al. 2007). Specific analyses of an RB gene expression signature in human patients indicate that deregulation of this pathway is associated with early recurrence following tamoxifen monotherapy. Thus, because the RB pathway is a critical determinant of tumorigenic proliferation and differential therapeutic response, it may represent a critical basis for directing therapy in the treatment of breast cancer. The RB tumor suppressor can be used as a biomarker for how tumors will respond to anti-estrogen therapy and could become the basis for deciding how patients with estrogen-receptor-positive breast cancer are treated clinically.

This is a way to predict when anti-estrogen drug therapies are inappropriate for patients with hormone-dependent breast cancer so that physicians can immediately begin treating the patient with alternative drugs that are more likely to succeed. However, comprehensive clinical research is needed before this new method for predicting the success of anti-estrogen drugs is applied in daily patient care.

Riboflavin Carrier Protein

Riboflavin carrier protein (RCP), serves to transport riboflavin to where it's needed throughout the body. RCP is estrogen modulated and is a growth and development protein that is synthesized and secreted by the liver. RCP plays a central role in pregnancy and infant development by transporting Riboflavin across the placenta to a fetus and from the mother to an infant. During pregnancy, RCP levels increase dramatically to support the needs of the fast growing cells. Serum RCP levels in cycling breast cancer patients are 3- to 4-fold higher than those in their normal counterparts. This difference in circulatory RCP levels between cancer patients and their age-matched normal counterparts is further magnified to 9- to 11-fold at the post-menopausal stage. In addition, there seems to be a good correlation between rising RCP levels and disease progression, since significantly higher RCP concentrations are encountered in patients with advanced metastasizing breast cancer versus those with early disease. Using specific MAbs, RCP could be localized immunohistochemically in the cytoplasm of invading neoplastic cells of lobular and ductal carcinomas of the breast, indicating that the malignant cells are probably the source of the elevated serum RCP levels in breast cancer. These findings suggest that measurement of circulatory RCP and the immunohistochemical staining pattern of RCP in biopsy specimens could be exploited as an additional biomarker in diagnosis/prognosis of breast cancer in women. RCP can serve as a specific biomarker for women related cancers in both the determination of presence and stage of the disease due to its estrogen driven relationship. Measurements conducted using radio immunoassay analysis have provided a reliable, sensitive and specific method to detect elevated levels of RCP in blood serum. The ELISA clinical diagnostic technique may well be suited for early detection of estrogen related cancers by measuring RCP.

Risk of Invasive Cancer after Diagnosis of Ductal Carcinoma In Situ

Biomarkers can identify which women who were initially diagnosed with ductal carcinoma in situ (DCIS) are at high or low risk of subsequent invasive cancer, whereas histopathology information cannot. A nested case control study in a population-based cohort of women who were diagnosed with DCIS and treated by lumpectomy alone showed that lesions that were p16+COX-2+Ki67+ or those detected by palpation were statistically significantly associated with subsequent invasive cancer (Kerlikowske et al. 2010). Eight-year risk of subsequent DCIS was highest for women with DCIS lesions that had disease-free margins of 1 mm or greater combined with either ER(−) ERBB2(+)Ki67(+) or p16(+)COX-2(−) Ki67(+) status. The finding will allow women with DCIS to be more selective about their course of treatment and, potentially, avoid aggressive forms of treatment such as complete mastectomy or radiation.

Serum CA 15-3 as Biomarker of Prognosis in Advanced Breast Cancer

Locally advanced breast cancer represents a heterogeneous subgroup of breast cancer with an often dismal outcome. Identifying prognostic factors has acquired great significance for the selection of optimal treatment in individual patients. Multimodality treatment options include chemotherapy followed by surgery, chemotherapy and radiotherapy; and addition of tamoxifen in hormone receptor-positive cases. In search for prognostic factors, baseline serum levels of carcinoembryonic antigen (CEA) and carbohydrate antigen (CA 15-3) have emerged as strong independent predictors of outcome in locally advanced breast cancer. High preoperative concentrations of CA 15-3 are associated with adverse patient outcome. These biomarkers can be added to other established prognostic factors such as postoperative nodal status, histological grade and response to adjuvant chemotherapy. Although CA 15-3 is used for monitoring therapy in advanced breast cancer, preoperative CA 15-3 levels may be combined with existing prognostic factors for predicting outcome in patients with newly diagnosed breast cancer.

Stage-Specific Embryonic Antigen-3

Breast cancer stem cells can be enriched by stage-specific embryonic antigen 3 (SSEA-3) and known protein markers (CD24 and CD44), and as few as 10 such enriched cells can develop tumor in mice (Cheung et al. 2016). The authors also showed that the enzyme galactosyltransferase (β3GalT5) for the biosynthesis of SSEA-3 is expressed in breast cancer stem cells and cancer cells but not in normal cells, and both SSEA-3 and β3GalT5 are shown to be essential for cancer cell survival. Suppression of SSEA-3 expression by knockdown of the gene encoding β-1,3-galactosyltransferase 5 (β3GalT5) in the globo-series pathway, led to apoptosis in cancer cells specifically but had no effect on normal cells. This finding is

further supported by the analysis of SSEA-3 and the two related globo-series epitopes SSEA4 and globo-H in stem cells (ESCs and iPSCs) and various normal as well as cancer cells, and by the antibody approach to target the globo-series glycans and the late-stage clinical trials of a breast cancer vaccine.

Suppressor of Deltex Protein

Suppressor of deltex protein (SDRP), a ubiquitin ligase, is a component of the Notch signaling pathway, which plays a key role in regulating cell proliferation. The human homolog of SDRP (hSDRP) is aberrantly expressed in ER-positive breast cancer cell lines. As a biomarker, hSDRP adds value to early diagnostic testing for breast cancer and has potential use for the stratification of breast cancer patient groups and improved targeting of the most appropriate treatment for each patient. Metastatic disease may be more common and aggressive in patients with cellular dysregulation relating to hSDRP in primary tumors. It is a target for therapeutic strategies for breast cancer management.

Tumor Microenvironment as Biomarker of Metastasis in Breast Cancer

Multiphoton-based intravital imaging has shown that invasive carcinoma cells in rat mammary tumors intravasate when associated with perivascular macrophages, identifying a potential tumor microenvironment of metastasis (TMEM). TMEM is defined as the tripartite arrangement of an invasive carcinoma cell, a macrophage, and an endothelial cell. In a case-control study of patients who developed metastatic breast cancer, TMEM density predicted the development of systemic, hematogenous metastases (Robinson et al. 2009). The ability of TMEM to predict distant metastasis was independent of lymph node status and other currently used prognostic factors. Quantitation of TMEM may be a useful new prognostic biomarker for breast cancer patients. This is the basis of a test for metastasis that most pathology laboratories can carry out. The test consists of a triple immunostain containing antibodies to the three cell types. A high number of TMEMs in a tissue sample means that the tumor is likely to metastasize or has already done so. This test could help doctors precisely identify patients that should receive aggressive therapy and might spare many women at low risk for metastatic disease from undergoing unnecessary and potentially dangerous treatment.

Type III TGF-β Receptor as Regulator of Cancer Progression

The TGF-β signaling pathway has a complex role in regulating mammary carcinogenesis and Type III TGF-β receptor (TβRIII), a ubiquitously expressed TGF-β coreceptor, acts as a genetic switch that regulates breast cancer progression and metastasis (Dong et al. 2007). Early in cancer, the protein acts as a tumor

suppressor, inhibiting the uncontrolled growth of cells. But as the cancer progresses, the protein switches sides and begins to promote the metastasis of cancer. Most human breast cancers lose TβRIII expression correlating with decreased TβRIII expression. TβRIII expression decreases during breast cancer progression, and low TβRIII levels predict decreased recurrence-free survival in breast cancer patients. Restoring TβRIII expression in breast cancer cells by administering the drug 5 aza-cytidine dramatically inhibits tumor invasion, angiogenesis, and metastasis. These results indicate that loss of TβRIII through allelic imbalance is a frequent genetic event during human breast cancer development that increases metastatic potential. If further studies confirm these findings, physicians could use the presence or absence of this receptor as a biomarker to identify women who should be treated more aggressively in an effort to eradicate their cancers before they spread. However, even the most aggressive chemotherapy treatments can leave behind errant cancer cells that later regrow and metastasize. To overcome this problem, it ultimately may prove possible to restore the TβRIII receptors in women prior to their receiving chemotherapy in order to inhibit the cancer's propensity to spread. Further studies are investigating whether measuring the levels of the TβRIII in cells can serve as a guide to making treatment decisions among cancer patients.

The fact that the flow cytometry approach enables one to determine the quantitative expression of important prognostic markers in breast cancer cells opens up unexpected possibilities for broad application of this technology in clinical samples obtained from needle biopsies or surgical biopsies of patients with breast cancer or with suspicion of breast cancers.

Diagnostic Tests Based on Breast Cancer Genes

Approximately 5–10% of cases of breast cancer are due to inheritance of a mutated copy of one of the two genes known as BRCA1 and BRCA2. The mutational spectra of BRCA1 and BRCA2 include many high penetrance, individually rare genomic rearrangements. Among patients with breast cancer and severe family histories of cancer who test negative (wild type) for BRCA1 and BRCA2, approximately 12% can be expected to carry a large genomic deletion or duplication in one of these genes. It is recommended that effective methods for identifying these mutations should be made available to women at high risk. A third gene mutation, CHEK2, is linked to high rates of breast cancer, although it is not as important as the BRCA1 and BRCA2 mutations in indicating breast cancer risk (Weischer et al. 2007). However, women may benefit from screening for the mutation, which was found in 1% of white, Northern European women. The study only included Danish women, leaving questions about its prevalence in black and Hispanic women unanswered. In the study, 0.5% of Danish women had the mutation and 12% of them developed breast cancer, compared to 5% of the women who did not carry the mutation. Women with the mutation who were over 60, overweight, and taking hormone replacement therapy had a 24% chance of developing breast cancer in 10 years.

BRACAnalysis (Myriad Genetics Inc), a test for hereditary breast and ovarian cancer, incorporates the most thorough full-sequence analysis for gene mutation detection ever employed on a broad commercial scale. Myriad and others have discovered and published information on an additional type of mutation, known as a large rearrangement that has not been detectable by commercial DNA sequencing technologies, but only by laborious, manual research-based methods. Such rearrangements are responsible for a small percentage of changes in the two breast cancer genes. Myriad added a panel of five common rearrangements to its BRACAnalysis test, accounting for nearly half of the total occurrence of large rearrangements in the two genes. Because large rearrangements are quite rare, a woman meeting the commonly employed selection criteria for BRACAnalysis has less than 0.5% risk of carrying one of the large rearrangement mutations. Myriad introduced BRACAnalysis Rearrangement Test (BART), a new automated molecular diagnostic test in the BRACAnalysis family of products. The added test detects rare, large rearrangements of the DNA in the BRCA1 and BRCA2 genes and is performed on women with exceptionally high risk who have tested negative for sequence mutations and the common large rearrangements already included in Myriad's test.

Another gene for breast and ovarian cancer has been identified, which explains the link between hereditary and "sporadic" (non-inherited) forms of these cancers. The gene has been named EMSY and maps to chromosome 11q13.5, a region known to be involved in breast and ovarian cancer. EMSY gene is amplified almost exclusively in sporadic breast cancer (13%) and higher-grade ovarian cancer (17%). In addition, EMSY amplification is associated with worse survival, particularly in node-negative breast cancer, suggesting that it may be of prognostic value. The remarkable clinical overlap between sporadic EMSY amplification and familial BRCA2 deletion implicates a BRCA2 pathway in sporadic breast and ovarian cancer.

Prognostic Role of Breast Cancer Genes

Three genes, homeobox 13 (HOXB13), interleukin-17B receptor (IL17BR) and CHDH, and the HOXB13:IL17BR ratio index in particular, strongly predict clinical outcome in breast cancer patients receiving tamoxifen monotherapy. HOXB13:IL17BR index is a strong independent prognostic factor for ER+ node-negative patients irrespective of tamoxifen therapy. These two biomarkers serve as the foundation of the Aviara Breast Cancer Index (bioMerieux), which offers a combined assessment of two biomarkers (H/I and MGI) for risk prognosis and treatment response prediction for chemo and hormonal therapy of breast cancer.

Activity of a gene, Dachshund (DACH1), which normally regulates eye development and development of other tissues, commandeers cancer-causing genes and returns them to normal. DACH1 inhibits the expression of the cyclin D1 gene, an oncogene that is overexpressed in about half of all breast cancers. Analysis of breast cancer patients has demonstrated that DACH1 correlates with tumor size, stage and metastasis, with its expression greatly reduced in metastatic breast cancer cells, but

increased nuclear DACH1 expression predicts improved patient survival. It can be used as a prognostic biomarker for breast cancer. Other cell fate-determining genes are being examined in an attempt to identify new therapeutics for breast cancer and metastasis.

Breast cells expressing high levels of p16 and/or COX-2, when coupled with proliferation, go on to become basal-like invasive tumors. These particular biomarkers indicate an abrogated response to cellular stress; cells overexpressing them that continue to proliferate have bypassed pRb-mediated signals to senesce. In contrast, cells with high p16 and/or COX-2, but low proliferation, have an intact Rb checkpoint and senescent program and do not go on to become carcinogenic. These biomarkers can be measured years before tumors actually arise, and thus can be used clinically to help dictate individualized treatment options for breast cancer.

Protein Biomarkers for Breast Cancer Prevention

Protein biomarkers suitable for the prevention of breast cancer must be extremely sensitive, easily detectable and highly correlated with the disease. They should be expressed in the reversible phase of carcinogenesis. Among the large number of candidate tumor-associated proteins, those related to the estrogen/chorionic gonadotropin/insulin pathway seem to be of most interest because these can be causally implicated. They presumably are the first to express differently and are open to hormonal treatments. The biomarkers that give information on membrane receptor-modulated signal transduction should be considered as well. Up to now, only tamoxifen has shown some preventive activity, suggesting that the estrogen pathway is useful indeed. Fenretinide and recombinant human chorionic gonatotropin (hCG) are also promising. But the financial requirements and the very long assessment periods largely prevent current research. Application of proteomics combined with bioinformatics can provide specific combinations of disease-related expression profiles that could identify high-risk groups with much more reliability and enable monitoring of preventive strategies.

Biomarkers to Evaluate Efficacy of Chemoprevention

Breast cancer chemoprevention studies are in progress with antiestrogens, retinoids and other drugs on preclinical models and on women with increased risk of developing breast cancer. It is still not known whether the above agents are efficacious in individual patients and which are the most reliable biomarkers to be assessed for efficacy. Over the past decade, researchers have developed short-term bioassays for efficacy in animals models of breast cancer that simulate the development and progression of human breast cancer. In these studies, they employed predominantly molecular biomarkers related to cell cycle progression, apoptosis and senescence. Tamoxifen, which has been widely used for treatment and more recently, for the prevention of breast cancer, may differentially affect cell proliferation and apoptosis

in mammary tumors and the expression levels of cyclin D1 and cyclin E might also be considered potential intermediate biomarkers of response of mammary tumors to tamoxifen and possibly to other selective estrogen receptor modulators. Other biomarkers are currently under investigation for assessment of the efficacy of various chemopreventive agents.

Biomarkers of Response to Chemotherapy of Breast Cancer

Biomarker Prognosis of Breast Cancer Treated with Doxorubicin A technology discovered at the NIH describes the identification of a manganese superoxide dismutase (MnSOD) polymorphism as a novel biomarker for the prognosis of doxorubicin therapeutic response in breast cancer patients, wherein a Val16Ala polymorphism of MnSOD is indicative of patient survival. More specifically, patients undergoing doxorubicin combination therapy with Val/Val, Val/Ala, and Ala/Ala genotypes had 95.2%, 79%, and 45.5% survival rates, respectively, in a case study of 70 unselected breast cancer patients. Carriers of the Ala/Ala genotype had a highly significantly poorer breast cancer-specific survival in a multivariate Cox regression analysis than carriers of the Val/Val genotype. This technology can be developed into an assay to screen for breast cancer patients who will be responsive to doxorubicin treatment. Further, as the MnSOD polymorphism is common in the population (15% to 20% of patients have the Ala/Ala genotype), it is a common risk factor for doxorubicin therapy. This technology can potentially be utilized as a screening tool applicable for all cancer types treated with doxorubicin and for personalizing treatment. Future studies include determining the mechanism in which the polymorphism modulates doxorubicin toxicity.

Decreased Breast Density as a Biomarker of Response to Tamoxifen Increased breast density on mammography is the leading risk factor for breast cancer, apart from age. The International Breast Intervention Study I (IBIS-I), a trial of tamoxifen for ER-positive breast cancer prevention conducted at the Cancer Research UK Centre for Epidemiology, Mathematics and Statistics in London has shown that a reduction in breast density of at least 10% may predict who benefits from the breast cancer preventive effects of tamoxifen. Those with reduced breast density after 12 to 18 months of treatment had a 52% reduced risk of breast cancer. By contrast, those women who did not have a decrease in breast density had only an 8% risk reduction.

Biomarkers to Predict Response or Resistance to Aromatase Inhibitors Aromatase inhibitors (AI) have been established as a useful hormonal therapy in hormone receptor-expressing breast carcinoma. However, changes in tumor protein expression after exposure to AIs are not well understood. These changes may provide insight into how breast carcinomas respond or develop resistance against AIs, and lead to the discovery of potential biomarkers to predict treatment responses. Among various protein biomarkers that were investigated, HSP70 demonstrated the most significant positive correlation with clinical response of the patients to AIs (Yiu et al. 2010).

Biomarker-Guided Decisions for Breast Cancer Therapy

According to American Society of Clinical Oncology Clinical Practice Guideline, in addition to ER, progesterone receptor and EGFR-2, there is sufficient evidence for clinical utility of the biomarker assays Oncotype DX, EndoPredict, PAM50, Breast Cancer Index, urokinase plasminogen activator, and plasminogen activator inhibitor type 1 in specific subgroups of breast cancer (Harris et al. 2016). No biomarker except for ER, progesterone receptor, and EGFR-2 was found to guide choices of specific treatment regimens, which should also consider disease stage, comorbidities, and patient preferences.

Research is needed in all areas in the guideline to continue to refine and redefine clinical utility of specific biomarkers. Inclusion of biomarker investigations at the beginning of clinical trials during conception and design and prospective or prospective-retrospective studies that validate the clinical utility of biomarker candidates are important for enabling selection of therapy for early-stage invasive breast cancer. Research also is needed to better understand the impact of age, race/ethnicity, and health disparities on the prognostic and predictive value of biomarker candidate.

Concluding Remarks and Future Prospects of Breast Cancer Biomarkers

Numerous biomarkers of breast cancer have been investigated but few have shown practical usefulness in management of patients. There is a need to start consolidating the pathways and analyzing overlapping/cooperative natures of molecules from pathways. Potential usefulness of the cytoplasmic kinases and coactivators, which may act as coregulators in the action of estrogen receptor, is likely to accelerate the development of the next generation of biomarkers for the surveillance, prognosis and therapeutic decisions for cancer.

Cervical Cancer Biomarkers

Cancer of the cervix is the second most common cancer in women. The mortality rates of cervical cancer could be drastically reduced by the implementation of population wide cytological screening test. Screening for cervical intraepithelial neoplasia (CIN) is usually performed by Pap smear or cervicovaginal lavage. Identification of women with abnormal cervical smears permits early treatment of lesions, but the high rate of false positive and negative results is a cause for concern. The oncogenic human papilloma virus (HPV) is the causal factor in the development of cervical cancer. The detection of the viral infection enables the identification of patients at risk, however, about 5–30% of the normal female population harbors these viruses and only very few of these develop clinically relevant lesions.

Digene Corp's Hybrid Capture (HC) 2 assay is used for molecular diagnosis of HPV. HC 2 assay has a greater sensitivity to detect CIN grade 3 or higher and its specificity is comparable to an additional cytologic test indicating atypical squamous cells of undetermined significance (ASCUS) or a more advanced lesion. Testing for high-risk HPV with the HC 2 test is useful in the detection of CIN grade 2/3 in low-grade CIN groups and in the selection of patients for colposcopy. Quest Laboratories now provides HC 2 test as a primary tool to detect cervical cancer along with Pap smears rather than as a secondary test. A growing body of data now demonstrates the ability of HPV testing to identify women at high risk of cervical cancer more accurately than the most advanced type of Pap. Some experts are recommending that the HPV test replace the Pap as the first-line tool for cervical cancer screening, particularly in low-resource countries in the developing world. HPV screening that distinguishes HPV16 and HPV18 from other oncogenic HPV types may identify women at the greatest risk of developing cervical cancer.

In advanced preneoplastic lesions HPV genomes are often integrated into cellular chromosomes. This leads to enhanced expression of the viral oncogenes. The detection of specific viral mRNA transcripts derived from integrated HPV genomes enables the identification of preneoplastic lesions with a particularly high risk for progression to invasive cancers (APOT-assay). These findings will enable establishment of highly sensitive, but specific and cost efficient new cancer early detection assays.

The activity of two viral oncogenes E6 and E7 initiates in a long-term process neoplastic transformation in few of the HPV harboring cells. As consequence of the expression of E7 a cellular marker protein (p16) is increasingly expressed in dysplastic cells. Monoclonal antibodies directed against p16 enable, therefore, to specifically identify dysplastic cells and derived invasive cancers in histological slides but also cytological smears by CINtec Assay (MTM Laboratories). Correlating the cancer-specific antigen to the histology and cytology, CINtec will provide more detailed and precise information for cancer screening and diagnostic to the examining pathologist. Clinical study data have already shown very promising results in its application for the early detection of cervical cancer. InPath System (Molecular Diagnostics Inc) uses a specific combination of protein-based markers that illuminate and map abnormal cells and is a useful method of screening for cervical cancer.

Exfoliated cervical cells are used in cytology-based cancer screening and may also be a source for molecular biomarkers indicative of neoplastic changes in the underlying tissue. However, because of keratinization and terminal differentiation it is not clear that these cells have an mRNA profile representative of cervical tissue, and that the profile can distinguish the lesions targeted for early detection used to Comparison of transcription profiles from samples of normal exfoliated cells and cervical tissue by whole genome microarrays show that the gene expression profile of exfoliated cervical cells partially represents that of tissue and is complex enough to provide potential differentiation between disease and healthy tissue. The findings encourage further exploration of gene expression using exfoliated cells to identify and validate applicable biomarkers.

Expression of human telomerase reverse transcriptase (hTERT) mRNA and protein have been investigated in cervix cancer, cervical intraepithelial neoplasia (CIN) and normal cervix. Upregulation of hTERT may play an important role in the development of CIN and cervix cancer, and could be used as an early diagnostic biomarker for cervix cancer.

Several studies have been presented evaluating p16INK4a as a potential biomarker for cervical cancer screening and diagnosis. CINtec p16INK4a-based immunocytochemistry protocols have been used on cervical cytology preparations. The results of the studies indicate that this approach improves the histological diagnosis of cervical cancer.

A metaanalysis has correlated AG and AA genotypes as well as A allele of IL-17A rs2275913 with an elevated risk of cervical cancer (Yang et al. 2017). Results show potential of IL-17A and IL-17F polymorphisms as predictive biomarkers for risk of cervical cancer.

Gastrointestinal Cancer Biomarkers

Two important cancers of the gastrointestinal system are esophageal cancer, gastric cancer and colorectal cancer (CRC). These will be described with regard to biomarker studies.

Esophageal Cancer Biomarkers

Carcinoma of the esophagus including carcinoma of gastroesophageal junction are rapidly increasing in incidence. Esophageal carcinogenesis is a multi-stage process, involving a variety of changes in gene expression and physiological structure change. Identification of dysplasia in mucosal biopsies is the most reliable pathologic indicator of an increased risk of development of squamous cell carcinoma and passes through the sequence of chronic esophagitis, low-grade and high-grade dysplasia and invasive carcinoma. Although Barrett's esophagus is a precursor to esophageal adenocarcinoma and has a well described sequence of carcinogenesis: the Barrett's metaplasia-dysplasia-adenocarcinoma sequence. Studies are in progress to discover biomarkers for risk of squamous cell carcinoma as well as the diagnosis and monitoring of the response to treatment.

Hypermethylation of several tumor suppressor genes is involved in the evolution and progression of esophageal adenocarcinomas (EAC). Efforts are now underway to develop noninvasive biomarkers for this disease. Hypermethylation of APC gene occurs in the plasma of 25% of EAC patients and this is significantly associated with reduced patient survival, suggesting that APC hypermethylation in the plasma may be a useful biomarker of biologically aggressive disease in EAC. Similarly 23% of EAC patients have hypermethylation of p16 gene in the serum DNA. Various studies have found the usefulness of analyzing methylation levels of p16, E-cad,

RARb, DAPK and APC in the peripheral blood not only as a screening and monitoring tool for EAC patients, but also as a biomarker of tumor recurrence (Hoffmann et al. 2009).

miRNA expression profiles of esophageal cancer reveal the oncogenic mechanism by miRNA-mediated post-transcriptional pathway. Further exploration is required for better understanding their role in carcinogenesis of esophageal cancer. Circulating miRNAs are potential biomarkers for esophageal cancer (Zhou and Wang 2010).

Gastric Cancer Biomarkers

While there is no reliable serum biomarker for the diagnosis and monitoring of patients with gastric cancer, proteomic technologies have been used extensively for detection of biomarkers of gastric cancer. An analysis of cryostat sections of central gastric tumor, tumor margin, and normal gastric epithelium using ProteinChip Arrays and SELDI-TOF MS revealed a peak that was significantly down-regulated in tumor tissue and identified as pepsinogen C using MS/MS analysis and immunodepletion (Melle et al. 2005). This signal was further characterized by immunohistochemistry. This work demonstrates that differentially expressed signals can be identified and assessed using a proteomic approach comprising tissue-microdissection, protein profiling, and immunohistochemistry. Pepsinogen C is a potential biomarker of gastric cancer.

Serum samples from patients with gastric cancer as well as healthy adults were examined by SELDI-TOF-MS and data of spectra were analyzed by Biomarker Patterns Software (Qian et al. 2005). Two mass peaks were selected as significant potential biomarkers. The sensitivity, specificity, and accuracy of the model were higher than those of clinically used serum biomarkers CEA, CA19-9 and CA72-4. Stage I/II gastric cancer samples of the test group were all judged correctly. The novel biomarkers in serum and the established model could be potentially used in the detection of gastric cancer. However, large-scale studies should be carried on to further explore the clinical impact on the model.

Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) is over-expressed in many human malignancies, including gastric cancer, and is associated with poor outcome. An elevated preoperative level of serum TIMP-1 is significantly associated with progressive disease, advanced stage, and worse survival in gastric cancer patients who under go surgery.

Aberrant DNA methylation is an early and frequent process in gastric carcinogenesis and could be useful for detection of gastric cancer. Six genes (MINT25, RORA, GDNF, ADAM23, PRDM5, MLF1) showed frequent differential methylation between gastric cancer and normal mucosa with close correlation between methylation levels in tumor biopsy and gastric washes (Watanabe et al. 2009). Of these MINT25 was found to be a sensitive and specific biomarker for screening in gastric cancer. Other genes that have been studied as biomarkers of gastric cancer

using methylation-specific PCR include: APC, DAPK, E-cad, GSTP1, MGMT, MLH1, MSP, p15, p16, Q-MSP, RUNX3, RARb, RASSF1A, SOCS1, TGFbRII, and TIMP3.

Real-time RT-PCR has shown that expression levels of miR-106a and miR-17 in preoperative and postoperative blood samples of patient with gastric carcinoma were significantly higher than those in controls; indicating that this may be a new tool for monitoring CTCs (Zhou et al. 2010a). In a similar study, plasma analyses for 5 selected miRNAs (miR-17-5p, miR-21, miR-106a, miR-106b and let-7a) were performed in gastric cancer patients and healthy volunteers (Tsujiura et al. 2010). The miRNAs were stable and detectable in all plasma samples, and the plasma miRNA levels reflected the tumor miRNAs in most cases. The levels of these miRNAs were significantly reduced in postoperative samples, indicating the prognostic value of miRNAs in gastric neoplasia. In large-scale analysis, the plasma concentrations of 4 of the 5 miRNAs (miR-17-5p, miR-21, miR-106a and miR-106b) were significantly higher in cancer patients than in controls, whereas mir-let-7a was lower in cancer patients. These results support the specificity and usefulness of this method detecting circulating miRNAs for diagnosis as well as prognosis.

Colorectal Cancer Biomarkers

CRC is one of the most common cancers in the world and is a leading cause of cancer mortality and morbidity. The cause of CRC is multifactorial, involving hereditary susceptibility, environmental factors, and somatic genetic changes during tumor progression. Detection of biomarkers is useful for prevention, diagnosis, prognosis and management of CRC. Biomarkers of CRC are listed in Table 13.9.

Detection of Serum Biomarkers of CRC One method for detecting serum biomarkers for CRC is by serum protein profiling using SELDI-TOF MS followed by classification tree pattern analysis. Biomarkers can be identified and reproducibly detected in independent sample sets with high sensitivity and specificity. Although not specific for CRC, these biomarkers have a potential role in monitoring the disease as well as the treatment. However, there is still a need for multiple biomarker testing and for identifying panels of predictive markers in order to improve response rates and decrease toxicity with the ultimate aim of tailoring treatment according to an individual patient and tumor profile. Soluble cytokeratin 18 fragment M65A is released from human cancer cells during cell death and hold potential as biomarkers in CRC characterized by frequent metastatic spread (Ausch et al. 2009).

BioServe and Phenomenome Discoveries Inc. (PDI) have developed a novel serum-based diagnostic test for the identification of CRC and pre-cancerous states conducive to the development of CRC. For developing the test, BioServe identified a large number of patient tissue and serum samples from its Global Repository exhibiting CRC across a spectrum of stages, as well as matched healthy controls. Using PDI's patented non-targeted metabolomics platform, PDI discovered that a

Table 13.9 Biomarkers of colorectal cancer

Aldo-keto reductase family 1 B10 (AKR1B10 or ARL-1)
Alpha-methylacyl-coenzyme A racemase (AMACR) in CRC tissue as prognostic biomarker
Biomarkers of resistance to chemotherapy: thymidylate synthase, topoisomerase-I, ABCB1/P-gp transporter
Cancer stem cell biomarkers in cancer
CRC-specific methylated DNA biomarkers in plasma: MLH1, p16, DAPK, TPEF/HPP1, APC, HLTF, ALX4, RUNX3, RASSF1A, RASSF2A, SEPT9, MGMT, and WIF1.
Circulating tumor cell biomarkers: Plastin3
Desmin
DNA microsatellite instability
Gene biomarkers of CRC
Guanylyl cyclase C
Insulin and insulin-like growth factor binding protein (IGFBP)-1
Matrix metalloproteinase 9
miRNA biomarkers of CRC
Mutations in DNA mismatch repair genes: hereditary nonpolyposis CRC
Serum CEA
Urinary biomarkers
Volatile organic compounds in exhaled breath

series of novel metabolites were significantly decreased in serum samples collected from CRC patients compared to controls. From these results, PDI developed a 2 min high-throughput screening method capable of simultaneously measuring a key subset of these molecules. The rapid test was found have a sensitivity of 75% and specificity of 90%. Trials are planned in Canada and Japan, in which health-care authorities will evaluate the test's utility as part of a broad-based population screening regimen.

Circulating Tumor Cell Biomarkers Circulating tumor cells (CTC) in blood are potential seeds for metastasis as well as cancer biomarkers. However, most CTC detection systems might miss epithelial-mesenchymal transition (EMT)-induced metastatic cells because detection is based on epithelial markers. Microarray analysis of CRC tissue specimens to detect genes that are overexpressed relative to normal colon mucosa led to discovery of plastin3 (PLS3) as a biomarker that is expressed in metastatic CRC cells but not in normal circulation (Yokobori et al. 2013). Fluorescent immunocytochemistry was used to validate that PLS3 was expressed in EMT-induced CTC in peripheral blood from patients with CRC with distant metastasis. PLS3 has also significant prognostic value.

Urinary Biomarkers of CRC Metabolomic research remains the primary means to identify urinary biomarkers in CRC. However, many serum biomarkers and tissue biomarkers are not excreted in the urine unless the plasma levels are high enough to overcome renal resorption. According to one study, 76.9% of CRC patients can be correctly classified using principal component analysis of 14 nucle-

osides by reversed-phase HPLC; 9 of these nucleosides were found to significantly decrease after curative resection thus implying prognostic value (Feng et al. 2009). This preliminary study indicates that the evaluation of both normal and modified urinary nucleosides might provide unique markers in the diagnosis and management of CRC.

miRNA Biomarkers of CRC CRC samples, characterized by microsatellite stability (MSS) as well as high microsatellite instability (MSI-H), have been investigated for genome-wide expression of miRNA and mRNA (Lanza et al. 2007). Based on combined miRNA and mRNA gene expression, a molecular signature consisting of differentially expressed genes including miRNAs, could correctly distinguish MSI-H versus MSS colon cancer samples. Among the differentially expressed miRNAs, various members of the oncogenic miR-17-92 family were significantly up-regulated in MSS cancers. The majority of protein coding genes were also upregulated in MSS cancers. Their functional classification revealed that they were most frequently associated with cell cycle, DNA replication, recombination, repair, gastrointestinal disease and immune response. This study suggests that the combination of mRNA/miRNA expression signatures may represent a general approach for improving biomolecular classification of human cancer. A study has shown that altered expression levels of miR-21, miR-31, miR-143 and miR-145 is associated with clinopathological features of CRC (Slaby et al. 2008).

Differential expression of specific miRNAs in tissues and blood offers the prospect of their use in early detection, screening and surveillance of CRC in a noninvasive manner.A study investigated whether plasma miRNAs could discriminate between patients with and without CRC (Ng et al. 2009). This study entailed an initial discovery of discriminatory miRNAs in a small subset of CRC patients versus normal subjects, followed by selection and validation of these miRNAs in an independent collection of plasma from patients with CRC, gastric cancer, and inflammatory bowel disease as well as healthy controls. Among the panel of 95 miRNAs analyzed, 5 miRNAs (miR-17-3p, miR-135b, miR-222, miR-92 and miR-95) were upregulated both in plasma and tissue samples. All 5 miRNAs were validated in the plasma of patients with CRC and healthy controls. Among these, two miRNAs, miR-17-3p and miR-92, were significantly elevated in the patients with CRC. The plasma levels of these 2 miRNAs were significantly reduced after surgery in patients with CRC. Further validation with an independent set of plasma samples indicated that miR-92 differentiated CRC from gastric cancer, IBD and normal subjects.

Expression of miR-31-3p in primary CRC samples is associated with anti-EGFR response in KRAS wild-type patients with the disease who have been treated with EGFR inhibitors. The miRpredX 31-3p test (IntegraGen) predicts clinical response to anti-EGFR therapy in RAS wild type patients with metastatic CRC assisting clinicians to identify the most appropriate therapeutic strategy when combining information on miRNA miR-31-3p expression with other molecular and patient factors which influence patient outcomes.

ColonSentry (GeneNews) is based on the Sentinel Principle™, which uses blood samples to identify RNA biomarkers for early diagnosis of CRC. It has been developed to provide:

- A simply convenient first step for CRC screening.
- High patient acceptance.
- To exploit genomics for CRC diagnostics.
- To enhance the number of cancers detected by colonoscopy.

Hereditary Nonpolyposis Colorectal Cancer (HNPCC) This is a familial cancer syndrome characterized by mutations in at least one of six DNA mismatch repair genes: hPMS1, hPMS2, hMSH2, MSH6, hTGFBR2 and hMLH1. From 5–10% of the 150,000 cases of CRC diagnosed each year in the US are of hereditary type. Identification of DNA microsatellite instability refines the diagnosis of HNPCC, allowing frequent early-onset colonoscopic screening to be restricted to individuals with an especially high risk of this type of cancer. Human homologues of murine miRNA sequences, miR-143 and miR-145, consistently display reduced steady-state levels of the mature miRNA at the adenomatous and cancer stages of colorectal neoplasia.

Diagnostic Biomarkers of CRC Increased levels of matrix metalloproteinase 9 (MMP-9), a biomarker of CRC that can be measured from a blood sample, is potentially an accurate, low risk and cost-effective population screening tool. The accuracy of serum MMP-9 as a test for CRC in a primary care population is being evaluated.

CRC-specific methylated DNA in plasma can be identified by use of high-performing biomarker assays (Lofton-Day et al. 2008). Restriction enzyme-based discovery methods were used to identify biomarker candidates with obviously different methylation patterns in CRC tissue and nonpathologic tissue. A selection process incorporating microarrays and/or real-time PCR analysis of tissue samples was used to further test biomarker candidates for maximum methylation in CRC tissue and minimum amplification in tissues from both healthy individuals and patients with other diseases. Three biomarkers, TMEFF2, NGFR, and SEPT9, were selected and tested with plasma samples. TMEFF2 methylation was detected in 65% of plasma samples from CRC patients and not detected in 69% of the controls. The corresponding results for NGFR were 51% and 84%; for SEPT9, the values were 69% and 86%. Application of stringent criteria at all steps of the selection and validation process enabled successful identification and ranking of blood-based biomarker candidates for CRC. The product, Epi proColon (Epigenomics Inc) blood test, offers an acceptable alternative to conventional screening methods for CRC.

A novel blood-based, 5-gene biomarker set has been reported for the detection of CRC (Han et al. 2008). Two of these were the most up-regulated (CDA and MGC20553) and three were the most down-regulated (BANK1, BCNP1, and MS4A1) in CRC patients. The predictive power of these five genes was validated with a novel third set, correctly identifying 88% of CRC samples and 64% of non-CRC samples.

Guanylyl cyclase C (GCC) is a cell surface molecule found on colorectal cells, both normal and cancerous, but not on any normal cells outside the intestine. GCC receptor provides a superior mechanism for detecting the presence of CRC cells because it relies on ultrasensitive mRNA-based amplification technology rather than other less sensitive and variable detection systems, such as the histopathology. Quantification of GCC mRNA in tissues by RT-PCR employing external calibration standards is analytically robust and reproducible, with high clinicopathologic sensitivity and specificity. GCC biomarker has shown to be 95% to 100% accurate in detecting the spread or recurrence of colon cancer in lymph nodes or blood. This is the basis for Signal Genetics' Previstage™ GCC (acquired by miRagen Therapeutics) as a lymph node test for the staging of CRC.

The aldo-keto reductase family 1 B10 (AKR1B10 or ARL-1) protein is normally expressed in mature epithelial cells of healthy colon tissue. AKR1B10 expression is noticeably decreased or absent in CRC and precancerous conditions. Scientists at the Southern Illinois University School of Medicine have developed a method to use AKR1B10 as a novel biomarker for CRC and other precancerous conditions. This biomarker would be useful in screening high risk populations, such as those with predisposing conditions of CRC, such as Crohn's disease, chronic inflammatory bowel disease, and ulcerative colitis.

Desmin, originally a tissue biomarker of heart failure, is found to be elevated in CRC tissue and fetal colorectal tissue compared with normal colorectal tissue. Desmin can be considered a potential serum biomarker for CRC that may have significance in the detection of patients with CRC (Ma et al. 2009).

Analysis of the volatile organic compounds (VOCs) linked to cancer is a new frontier in cancer screening, as tumor growth involves several metabolic changes leading to the production of specific compounds that can be detected in exhaled breath. Canine scent detection has shown that odor of VOCs is an effective tool in CRC screening. A study investigated whether patients with CRC have a specific VOC pattern compared with the healthy population (Altomare et al. 2013). Exhaled breath was collected in an inert bag (Tedlar®) from patients with CRC and healthy controls (negative colonoscopy), and processed offline by thermal-desorber gas chromatography-MS to evaluate the VOC profile. During the trial phase VOCs of interest were identified and selected, and VOC patterns able to discriminate patients from controls were set up; in the validation phase their discriminant performance was tested on blinded samples. A probabilistic neural network (PNN) validated by the leave-one-out method was used to identify the pattern of VOCs that better discriminated between the two groups. Application of a PNN to a pattern of 15 compounds showed a discriminant performance with a sensitivity of 86%, a specificity of 83% and an overall accuracy of 76%. The pattern of VOCs in patients with CRC was different from that in healthy controls. Breath VOC analysis appears to have potential clinical application as a biomarker in CRC screening, although further studies are required to confirm its reliability in heterogeneous clinical settings.

Biomarkers for Prevention and Management of CRC A case-control study, nested within the European Prospective Investigation into Cancer and Nutrition,

confirmed a role for the BsmI polymorphism of the VDR gene in CRC risk, independent of serum 25OHD concentration and dietary calcium intake (Jenab et al. 2009). It is possible that a combination of tests for microsatellite instability, allelic loss, p53 mutations, and other genetic alterations in patients with early stage CRC will define groups of patients who require different adjuvant therapies or no systemic treatment at all. Serum CEA has also been used as a biomarker for surveillance and monitoring of therapy of CRC. Clinical use of targeted therapy is hampered by several questions that need to be answered such as optimal biologic dose and schedule, lack of predictive surrogate biomarkers, and modalities of combination with chemotherapy/radiotherapy. To answer these, high throughput methods are in use to discover prognostic and predictive markers for CRC.

Biomarkers of Survival in CRC Location and amount of thymidylate synthase (TS) within 2 separate compartments of a tumor cell may be critical biomarkers pfor redicting survival in CRC. AQUA™ technology (Navigate BioPharma Services Inc) that combines fluorescence-based imaging, microscopy and high-throughput tissue microarray technologies, has been used to determine subcellular TS levels. High levels of the protein in the nucleus correlate with decreased patient survival time, and, further, a high ratio of TS in the nucleus relative to the level in the cytoplasm correlates with a shorter survival time. Thus, relationship between nuclear and cytoplasmic levels of TS can predict survival, although TS levels were used previously as a biomarker for decreased survival and response to therapy.

Obesity, sedentary lifestyle, and Western dietary pattern have been linked to increased risk of cancer recurrence and mortality among patients with surgically resected CRC. Excess energy balance leads to increased circulating insulin and depressed levels of circulating insulin-like growth factor binding protein (IGFBP)-1, which promote cancer cell growth in preclinical models. Thus, circulating insulin and IGFBP-1 are potential mediators of the association between lifestyle factors and mortality after CRC resection. Blood levels of these two insulin-related proteins can be used as biomarkers to predict which patients with CRC are most likely to die of their disease. In a study on patients with surgically resected CRC, higher levels of prediagnosis plasma C-peptide and lower levels of prediagnosis plasma IGFBP-1 were associated with increased mortality (Wolpin et al. 2009). Those with the highest levels of plasma C-peptide had an 87% percent greater chance of dying overall and a 50% percent greater chance of dying from CRC than those with the lowest levels. The difference may be due to the fact that C-peptide is basically insulin, which is associated with diseases of the heart and other systems.

Men consuming high amounts of red meat and dairy products are considered to be at higher risk of developing CRC and prostate cancer. Alpha-methylacyl-coenzyme A racemase (AMACR) is an enzyme that helps to break down fat from these foods to produce energy. An increase in the utilization of energy from fat is a hallmark of many cancers. AMACR is also highly expressed in certain stages of CRC and a close examination of the this gene in a panel of normal and progressively malignant colon tissues reveals that deletions of specific sequences in the AMACR gene may trigger its abnormal expression during the evolution of CRC (Zhang et al. 2009).

A new deletion variant of the AMACR gene may serve as a biomarker of prognosis and survival in CRC.

CSC-associated biomarkers have potential for determining prognosis of CRC (Langan et al. 2013). These include CD133, CD24, CD29, EpCAM, and ALDH1B1.

Biomarkers of Resistance to Chemotherapy Despite the recent results of systemic chemotherapy, more than 40% of patients with advanced cancer still do not achieve substantial benefits with cytotoxic agents. Resistance to chemotherapy is an important factor in poor response to treatment. Mechanisms that may have important implications for drug efficacy and actively contribute to innate resistance in CRC are:

- High levels of TS, the 5-FU target, are associated with tumor insensitivity to FU-based therapy.
- Higher levels of topoisomerase-I (TOP1) correlate with greater sensitivity of colon tumors to camptothecin derivatives compared to normal colonic mucosa.
- Glucuronidation, involved in xenobiotic detoxification, is also associated with innate resistance to TOP1 inhibitors in colon cell lines and tumors.
- An increase of the ABCB1/P-gp transporter, a member of the family of ABC-transporters that detect and eject anticancer drugs from cells, is observed in intrinsically drug-resistant colon tumors.

The success of chemotherapy depends on various factors such as gender, age and histological subtype of tumor. The difference in drug effects between different genotypes can be significant. Promising candidates have been identified with predictive value for response and toxicity to chemotherapy in CRC. These candidates need to be incorporated into large, prospective clinical trials to confirm their impact for response and survival to chemotherapy that has been reported in retrospective analyses. Confirmed predictive markers, together with additional yet to be identified pharmacogenomic key players, will provide the basis for tailoring chemotherapy in the future. The rationale for this approach is based on the identification of the in vivo interactions among patient's characteristics, disease physiopathology, and drug pharmacodynamics as well as pharmacokinetics.

OncoTrack to Develop Biomarkers for Personalized Management of CRC In 2011, OncoTrack, an international consortium of >80 academic researchers, pharmaceutical companies, and commercial partners, launched a 5-year project to develop and assess new biomarkers for CRC. Total budget for the project along with funding from the Innovative Medicines Initiative — a private-public partnership between the pharmaceutical industry and the European Union — amounted to ~$35.6 million. OncoTrack was founded to create next-generation methods of biomarker development for personalized treatment of CRC. The consortium, led by Bayer AG and the Max Planck Institute for Molecular Genetics in Germany, includes AstraZeneca, Boehringer Ingelheim, Janssen Pharmaceutica, Merck, Pfizer, and Roche Diagnostics. Academic partners include Uppsala University, University College London, Paris South University, Charité Universitätsmedizin Berlin, Medizinische Universität Graz, and Technische Universität Dresden.

International Prevention Research Institute, Experimental Pharmacology and Oncology, and Alacris Theranostics also are members of OncoTrack. The consortium's first project called "Methods for systematic next-generation oncology biomarker development" started to generate high-quality genomic and epigenetic data from clinically well-defined CRC tumors and their metastases. The data will be compared to the germline genome of the patients, and will be complemented by a detailed molecular characterization of the tumors. Cutting edge laboratory-based genome sequencing techniques coupled to novel computer modelling approaches will be used to study both the patient to patient variability as well as tumor variation within the patient, e.g., by comparing primary tumors with metastases.. The combined data from all phases of the project will enable OncoTrack to address fundamental questions regarding the relationship between tumor genotype and phenotype, thus providing the starting point for discovery and selection of suitable candidates for development as biomarkers of CRC to guide patient therapy, provide immediate feedback upon the effects of treatment and ultimately indicate likely outcome of disease management. This comprehensive mapping of the CRC molecular landscape along with clinical functional annotation for systems biology analysis provides unprecedented insight and predictive power for personalized management of CRC (Henderson et al. 2014). The results have not been published as yet.

Concluding Remarks on Biomarkers of CRC The American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology convened an expert panel to develop an evidence-based guideline to establish standard molecular biomarker testing and guide therapies for patients with CRC. Important conclusions were as follows (Sepulveda et al. 2017):

- Mutational testing of specific genes in the EGFR signaling pathway provides clinically actionable information for targeted therapy of CRC with anti-EGFR MAbs.
- Mutations in some of the biomarkers have clear prognostic value (BRAF, MMR), and at least two (KRAS and NRAS) have relatively strong evidence as negative predictors of benefit to anti-EGFR therapies and should be used to guide the use of these agents. BRAF mutations are consistently associated with poor outcomes in patients with metastatic CRC, including those who relapse after adjuvant therapy. Patients with localized colon cancer and dMMR have improved outcomes. MMR status has predictive value in some settings, specifically in patients with advanced disease being considered for anti-PD-1/PD-L1 therapy.
- Whereas earlier testing approaches were focused on one or a few testing targets (e.g., BRAF p.V600 mutations), new approaches use gene panels such as targeted NGS cancer panels, which can range from a few to hundreds of genes and amplicons with known mutational hotspots in cancer.

Head and Neck Cancer

Head and neck squamous cell carcinoma (HNSCC) is a leading cause of cancer mortality worldwide. Gene expression signatures generated from DNA microarray analyses using formalin-fixed HNSCC tumors have shown that genes involved in epithelial-to-mesenchymal transition (EMT) and nuclear factor-kappaB (NF-κB) signaling deregulation are the most prominent molecular characteristics of the high-risk tumors. The difference in recurrence-free survival between the high-risk versus low-risk groups was statistically significant. The 75-gene list, determined by training on the formalin-fixed tumor data set and tested on data from the independent frozen tumor set, can be used as a prognostic biomarker of recurrence. These data suggest that the molecular determinants of EMT and NF-κB activation can be targeted as the novel therapy in the identified high-risk patients.

A subset of HNSCC have been associated with high-risk human papillomaviruses (HPVs), particularly HPV16, the same subset of HPVs responsible for the majority of cervical and anogenital cancers. In a transgenic mouse model for HPV-associated HNSCC, HPV16 oncogenes mirrors the molecular and histopathological characteristics of human HPV-positive HNSCC that distinguish the latter from human HPV-negative HNSCC. This validated model provides the means to define the contributions of individual HPV oncogenes to HNSCC and to understand the molecular basis for the differing clinical manifestations of HPV-positive and HPV-negative human HNSCC. This study identified minichromosome maintenance protein 7 (MCM7) and p16 as potentially useful biomarkers for HPV-positive head and neck cancer. However, recent studies have shown that a positive test for HPV DNA alone is not significantly linked to head and neck cancer outcomes. On the other hand, when found in combination with E6 and E7 expression, a positive HPV16 test did coincide with improved oropharyngeal cancer outcomes. Likewise, elevated levels of p16 in a tumor were not especially informative on their own, though they do correspond to better oropharyngeal cancer survival when found together with positive blood tests for E6 and E7.

A positive test for HPV DNA alone was not significantly linked to head and neck squamous cell carcinoma (HNSCC) outcomes. On the other hand, when found in combination with E6 and E7 expression, a positive HPV16 test did coincide with improved oropharyngeal cancer outcomes. Likewise, elevated levels of p16 in a tumor were not especially informative on their own, though they did correspond to better oropharyngeal cancer survival when found together with positive blood tests for E6 and E7. Based on these findings, it is concluded that a stronger association of HPV presence with prognosis (assessed by all-cause survival) is observed when HPV-associated HNSCC is defined using tumor status (HPV DNA or P16) and HPV E6/E7 serology in combination rather than using tumor HPV status alone (Liang et al. 2012).

Another study on oropharyngeal squamous cell carcinomas (OPSCC) found its own evidence arguing against the use of HPV DNA as a solo biomarker for

HPV-associated cancer (Holzinger et al. 2012). They tested OPSCC tumors for HPV DNA and p16. They also considered the viral load in the tumors and looked for gene expression profiles resembling those described in cervical carcinoma, another cancer associated with HPV infection. Again, the presence of HPV DNA appeared to be a poor indicator of HPV-associated cancers or predictor of cancer outcomes. Whereas nearly half of the tumors tested positive for HPV16 DNA, just 16% and 20% had high viral loads and cervical cancer-like expression profiles, respectively. The researchers found that a subset of HPV DNA-positive tumors with high viral load or HPV-associated expression patterns belonged to individuals with better outcomes. In particular, they found that cervical cancer-like expression profiles in OPSCC tumors coincided with the most favorable outcomes, while high viral load in the tumors came a close second. Once standardized assays for these biomarkers, applicable in routine clinical laboratories, are established, they will allow precise identification of patients with oropharyngeal cancer with or without HPV-driven cancers and, thus, will influence prognosis and potentially treatment decisions. More research is needed to understand whether the patterns described in the new studies hold in other populations and to tease apart the prognostic importance of HPV infection in relation to additional prognostic biomarkers.

The proto-oncogene pituitary tumor-transforming gene (PTTG) has been shown to be abundantly overexpressed in a large variety of neoplasms likely promoting neovascularization and tumor invasiveness. Elevated PTTG transcript levels may be used as a prognostic biomarker for future clinical outcome (i.e. recurrence) in primary squamous cell carcinomas of the head and neck, especially in early stages.

EGFR is another promising biomarker for HNSCC but further research is required to determine its prognostic benefit. Several promising biomarker candidates are now being evaluated, including epigenetic, expression and genomic-based biomarkers. Studies to validate the sensitivity and specificity of these biomarkers in clinical samples from adequately powered prospective cohorts are needed for successful translation of these findings into potential molecular diagnostic, prognostic and therapeutic biomarkers for HNSCC (Mydlarz et al. 2010).

A close look at the HNSCC transcriptome analyses has revealed some genes that are frequently dysregulated and are specific candidates as HNSCC molecular biomarkers (Lallemant et al. 2009). Nine genes displaying frequent alterations in HNSCC are FN1, MMP1, PLAU, SPARC, IL1RN, KRT4, KRT13, MAL, and TGM3. MMP1 detection in saliva rinse is potentially useful for non-invasive diagnosis of HNSCC of the oral cavity or oropharynx with 100% specificity, but technical improvement is needed since sensitivity is only 20%. IL1RN, MAL and MMP1 are prospective tumor diagnostic biomarkers for HNSCC. MMP1 overexpression is the most promising biomarker, and its detection could help identify tumor cells in tissue or saliva.

Leukemia Biomarkers

Conventional methods for the diagnosis of leukemias are blood counts with staining and examination of cells, examination of bone marrow following aspiration, biochemical screening, chromosome analysis of cells to detect dislocations and immunophenotyping. Molecular diagnostics is now applied for assessment of leukemias. Various biomarkers of leukemias are described here fall into the following categories:

- Chromosome translocations in leukemias
- Gene mutations
- Proteins
- miRNA biomarkers

Chromosome Translocations in Leukemias

Chromosome translocations (rearrangements), which are present in most human leukemias, are widely used by clinicians as diagnostic and prognostic tools. At the molecular level, translocations are especially valuable because they immediately indicate the spot in which to search for a cancer gene. Chromosome breakpoints can now be cloned and sequenced efficiently, and the relevant genes can be rapidly identified. The t(9;22) translocation known as the Philadelphia chromosome was the first tumor-specific cytogenetic marker identified in a human cancer. Its discovery eventually led to the cloning of the BCR-ABL fusion region. The presence of a Philadelphia chromosome confers a poor prognosis in cases of acute lymphoblastic leukemia (ALL). Rearrangements of the MLL (mixed lineage leukemia) gene located at chromosome band 11q23 are commonly involved in adult and pediatric cases of primary acute leukemias and also found in cases of therapy-related secondary leukemias. Approximately 50 different chromosomal translocations of the human MLL gene are currently known and associated with high-risk acute leukemia. The large number of different MLL translocation partner genes makes a precise diagnosis a demanding task. After their cytogenetic identification, only the most common MLL translocations are investigated by RT-PCR analyses, whereas infrequent or unknown MLL translocations are excluded from further analyses. To overcome this limitation, a universal long-distance inverse-PCR approach was devised that enables the identification of any kind of MLL rearrangement if located within the breakpoint cluster region (Meyer et al. 2005). This diagnostic tool has been proven successful for analyzing any MLL rearrangement including previously unrecognized partner genes. Furthermore, the determined patient-specific fusion sequences are useful for minimal residual disease monitoring of MLL associated acute leukemias.

Various specific chromosome rearrangements, including t(8;21), t(15;17), and inv(16), are found in acute myeloid leukemia (AML) and in childhood acute

lymphocytic leukemia (ALL), t(12;21) and t(1;19) are common. Most childhood leukemias begin before birth and that maternal and perinatal exposures such as chemical and infectious agents are likely to be critical. Epigenetic events are also important in the development of some forms of childhood leukemia. Some studies now show that the inactivating NAD(P)H:quinone acceptor oxidoreductase (NQO1) C609T polymorphism is positively associated with leukemias arising in the first 1–2 years of life and polymorphisms in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene have been associated with adult and childhood ALL.

DNA Methylation Biomarkers in Leukemia

Several studies have shown that methylated DNA biomarkers can be used to detect leukemia and other hematological malignancies. Histone H3 methylation may be changed dramatically during normal cell differentiation. The residual histone H3 methylation in myeloid leukemia cells suggests an incomplete chromatin condensation that may be linked to the leukemia cell proliferation and may be important for the prognosis of disease. Advantages of study of methylation biomarkers in leukemia are:

- Sensitivity. Detection of DNA in blood eliminates need for tissue biopsy
- Ease of use. DNA is a stable material for clinical assay.
- Cost effective. Can replace need to perform medical imaging or tissue biopsy.
- Multiple uses. Diagnosis, treatment monitoring and detecting disease relapse.

Gene Mutations as Biomarkers in Leukemia

Patients with AML and normal karyotype constitute the single largest cytogenetic group of AML, estimated to account for 45% of adults with de novo AML. Four prognostic biomarkers-the internal tandem duplication and point mutations in the FLT3 gene, partial tandem duplication of the MLL gene, mutations of the CEBPA gene, and overexpression of the BAALC gene-have been reported to predict outcome in patients with AML and normal cytogenetics. Because mutations in FLT3 result in an autophosphorylated, leukemogenesis-driving protein, molecular targeting therapy with a new class of tyrosine kinase inhibitors is being explored in early clinical trials. Considerable progress has been made in molecular characterization of AML patients with normal cytogenetics. The challenge for the future is to incorporate these biomarker discoveries into novel risk-adapted therapeutic strategies that will improve the currently disappointing cure rate (approximately 25–40%) of this group of patients.

Genzyme Diagnostics has launched two molecular tests for AML: FLT3 Mutation Analysis and WT1 RQ-PCR. FLT3 mutations are considered a prognostic indicator of poor survival and response to standard chemotherapies. Approximately 30% of patients with AML have FLT3 mutations. WT1 RQ-PCR test is designed to detect

MRD or very low levels of disease. The WT1 gene is expressed in approximately 90% of patients with AML. This test allows physicians to monitor AML patients for early relapse during and following therapy. Both of these tests may enable oncologists to better manage their patients.

Molecular Diagnostic Techniques for Leukemia

Molecular probes are used to diagnose acute or chronic leukemia. Both DNA mapping by Southern blotting and PCR can be used to detect chromosomal translocations (e.g. the Philadelphia chromosome) and determine the type of rearrangement. BCR-ABL translocation can be detected and quantified with the use of mRNA down to a level of 10^{-6} (i.e. 1 leukemia cell per 10^6 total cells). Light microscopy, cytogenetic analysis, flow cytometry, in situ hybridization, and Southern blot analysis will not detect malignant cells until their population exceeds 1–5% of the normal cell total. By contrast, PCR is sufficiently sensitive to detect one leukemia cell among 100,000 or even 1 million normal cells.

FISH probes directed against the BCR and ABL genes can reliably detect the fusion gene with a sensitivity of 0.05%. Reverse transcriptase PCR (rt-PCR) is capable of detecting very low levels of BCR-ABL mRNA transcripts allowing detection of a single leukemia cell. Real time PCR can quantify changes in transcript number and thus levels of residual disease and is useful in guiding clinical decision making. This is particularly useful in detecting relapse following allogeneic stem cell transplantation and in predicting the likelihood of a durable complete cytogenetic response to interferon. Patients who become 100% Ph negative on interferon, as detected by routine cytogenetic evaluation of 20 cells (20% or less of interferon treated patients), have a wide range of levels of PCR positivity. Only those with low levels of BCR-ABL transcripts will remain cytogenetically negative for prolonged periods.

Infants and children diagnosed with chemotherapy resistant acute lymphoblastic leukemia may in fact have a different type of leukemia. Gene chip technology has been used successfully to categorize 95% of the leukemias as ALL, AML or MLL. The signatures provided by RNA profiling might have enough information content to enable not only to determine prognosis but will facilitate stratification of patients into tailored therapeutic strategies.

Proteomic Technologies for Discovering Biomarkers of Leukemia

Proteomics is being used to subclassify leukemia, because cytogenetic analysis is costly and time-consuming. Several proteins have been identified that may serve as useful biomarkers for rapidly identifying different forms of childhood leukemia. Proteomic analysis of different subtypes of AML cells, carried out using 2D GE and MALDI-TOF analysis, identified proteins that are more significantly altered belonged to the group of suppressor genes, metabolic enzymes, antioxidants,

structural proteins and signal transduction mediators. Among them, seven identified proteins are significantly altered in almost all the AML blast cells analyzed in relation to normal mononuclear blood cells: alpha-enolase, RhoGDI2, annexin A10, catalase, peroxiredoxin 2, tromomyosin 3, and lipocortin 1 (annexin 1). These differentially expressed proteins are known to play important roles in cellular functions such as glycolysis, tumor suppression, apoptosis, angiogenesis and metastasis, and they might contribute to the adverse evolution of the disease. Using similar proteomic techniques other proteins have been identified that are expressed differentially in AML: alpha-2-HS-glycoprotein, complement-associated protein SP-40, RBP4 gene product, and lipoprotein C-III are downregulated, whereas immunoglobulin heavy-chain variant, proteosome 26S ATPase subunit 1, and haptoglobin-1 are upregulated. Proteomic analysis has identified novel proteins that may either help to determine a differential prognosis or be used as biomarkers for disease outcome, thus providing potential new targets for rational pathogenesis-based therapies of AML.

Biomarkers of Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL), which which is usually diagnosed at early stages, has an extremely variable individual prognosis, as some patients remain stable for years whereas others rapidly develop aggressive forms of the disease. Biomarkers provide useful information for prognosis. These include cytogenetical abnormalities diagnosed by FISH, mutational status of the immunoglobulin genes (IgVH) and expression of zeta-associated protein 70 (ZAP-70). Some notable findings are (Véronèse et al. 2008; Gribben 2008):

- The detection of del 17p or del 11q is associated with poor risk, while del 13q as a sole abnormality is associated with good-risk disease.
- Nearly half of CLL cases have somatic hypermutation in the variable regions of the genes and this has prognostic significance.
- Some studies have suggested that ZAP70 status is more useful as a predictor of time to progression than mutation status, but this remains controversial.
- Most of the modern prognostic markers were validated by retrospective analysis, and have now been applied to prospective randomized clinical trials. These studies suggest that the same molecular biomarkers that identify patients with more aggressive disease also impact on outcome after treatment.

Several CLL risk factor biomarkers (immunoglobulin heavy chain variable region mutational status, CD38 and ZAP-70 expression, CCL3 plasma levels) are related to to B-cell receptor (BCR) function and signaling, a key factor in CLL pathogenesis (Burger 2012). With the availability of inhibitors of BCR-associated kinases (Btk-, Syk- and PI3 kinase inhibitors) in CLL and other B cell malignancies, BCR-related risk factors may help with identification of patients who are likely to respond and/or for response assessment (i.e. CCL3 plasma levels).

A study has shown that circulating miRNAs can be sensitive biomarkers for CLL, because certain extracellular miRNAs are present in CLL patient plasma at levels significantly different from healthy controls and from patients affected by other hematologic malignancies. The levels of several of these circulating miRNAs also displayed significant differences between ZAP-70+ and ZAP-70− CLL (Moussay et al. 2011). The authors also determined that the level of circulating miR-20a correlates reliably with diagnosis-to-treatment time. Network analysis of their data suggests a regulatory network associated with BCL2 and ZAP-70 expression in CLL suggesting the possibility of using the levels of specific miRNAs in plasma to detect CLL as well as to determine the ZAP-70 status.

Biomarkers of Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a clonal multi-step myeloproliferative disease that is initially produced and ultimately sustained by a rare subpopulation of BCR-ABL+ cells with multi-lineage stem cell properties. These BCR-ABL+ CML stem cells are phenotypically similar to normal hematopoietic stem cells which are also maintained throughout the course of the disease at varying levels in different patients. Defining the unique properties of the leukemic stem cells that produce the chronic phase of CML has therefore had to rely heavily on access to samples from rare patients in which the stem cell compartment is dominated by leukemic elements. A study has reviewed past and ongoing approaches using such samples to identify biologically and clinically relevant biomarkers of BCR-ABL+ stem cells that explain their unusual biology and that may help to design, or at least predict, improved treatment responses in CML patients (Jiang et al. 2008).

Genomewide expression profiling of miRNAs can be carried out in CML by using a microarray containing hundreds of human precursor and mature miRNA oligonucleotide probes. miRNA expression profiles can be used to distinguish normal B cells from malignant B cells in patients with CLL. Mutations in miRNA transcripts are common and may have functional importance. A unique miRNA signature is associated with prognostic factors and disease progression in CLL.

Biomarkers of Drug Resistance in Leukemia

It is unclear why some patients develop resistance to imatinib mesylate or other anti-cancer agents, and what can be done to prevent or delay the onset of resistance. With regard to imatinib, resistance has been associated with several mechanisms including; (1) amplification or mutations of the BCR-ABL fusiongene; (2) inactivation by binding to α-1 acid glycoprotein; and (3) increased usage of signal transduction pathways that are BCR-ABL independent. However, these pathways remain undefined.

Biomarkers of Myelodysplasitic Syndromes

Myelodysplastic syndromes (MDS) are among the most frequent hematologic malignancies. Patients have a short survival and often progress to acute myeloid leukemia. The diagnosis of MDS can be difficult; there is a paucity of molecular markers, and the pathophysiology is largely unknown. A multicenter study was conducted to investigate whether serum proteome profiling may serve as a noninvasive platform to discover novel molecular biomarkers for MDS (Aivado et al. 2007). Peptide mass fingerprinting and quadrupole TOF MS identified two differential proteins: CXC chemokine ligands 4 (CXCL4) and 7 (CXCL7), both of which had significantly decreased serum levels in MDS, as confirmed with independent antibody assays revealed that these two proteins have decreased serum levels in advanced MDS, suggesting the possibility of a concerted disturbance of transcription or translation of these chemokines in advanced MDS.

Lymphoma Biomarkers

Lymphoma is a cancer that begins in the lymphatic cells of the immune system and presents as a solid tumor of lymphoid cells. There are three large groups: the B cell (including lymphocytic leukemia described in previous section under leukemias), T cell, and natural killer cell tumors. Novel biomarkers for categorization or risk stratification in patients with diffuse large B-cell lymphoma are being developed and validated. Hodgkin's lymphomais a tumor of abnormal lymphocytes of mature B cell lineage.

Several HDAC inhibitors are in development, many of which have been shown preclinically to have potent antitumor activity. Clinical trials using these agents are now underway, with Vorinostat (suberoylanilide hydroxamic acid) having been approved by the FDA for treating cutaneous T-cell lymphoma (CTCL) in patients with progressive, persistent or recurrent disease. HR23B is a candidate cancer biomarker identified in a genome-wide loss-of-function screen which influences sensitivity to HDAC inhibitors. A study has shown that HR23B governs the sensitivity of CTCL cells to HDAC inhibitors (Khan et al. 2010). Furthermore, proteasome activity is deregulated in HDAC inhibitor-treated CTCL cells through a mechanism dependent upon HR23B, and HDAC inhibitors sensitize CTCL cells to the effects of proteasome inhibitors. The predictive power of HR23B for clinical response to HDAC inhibitors was investigated through an analysis of a unique collection of CTCL biopsies taken from a phase II clinical trial, where there was a frequent coincidence between HR23B expression and clinical response to HDAC inhibitor, supporting the biomarker-based approach to personalized treatment of cancer.

A compendium of N-glycoproteins, which are potential cancer biomarkers, has been generated from human primary lymphomas and cell lines by using mass spectrometry (Rolland et al. 2017). N-glycoproteins are therapeutic targets for small molecules, antibodies, and cellular therapies. In anaplastic lymphoma kinase-

positive (ALK+) anaplastic large cell lymphoma, integration of N-glycoproteomics and transcriptome sequencing revealed an ALK-regulated cytokine/receptor signaling network, including vulnerabilities corroborated by a genome-wide clustered regularly interspaced short palindromic screen. These results highlight the utility of functional proteogenomic approaches for discovery of cancer biomarkers and therapeutic targets.

Liver Cancer Biomarkers

Liver cancer is the fifth most common cancer in the world and one of the deadliest cancers since it is rarely diagnosed until late in its development. The lack of reliable screening tests for liver cancer contributes to its high mortality rate since tumors seldom cause symptoms until the later stages when treatment options become limited and the prognosis is poorer. Death usually occurs not long after diagnosis.

It is important to detect hepatocellular carcinoma (HCC) and the recurrence at its earlier period. Serum tumor biomarkers for detecting HCC can be divided into 4 categories: oncofetal antigens and glycoprotein antigens; enzymes and isoenzymes; genes; and cytokines. Serum alpha fetoprotein (AFP) is the most widely used tumor biomarker in detecting patients with HCC. Some tumor biomarkers, such as human cervical cancer oncogene and human telomerase reverse transcriptase mRNA, are considered to be more accurate than AFP. Other tumor biomarkers, such as glypican-3, gamma-glutamyl transferase II, alpha-l-fucosidase, transforming growth factor-beta1 (TGF-β1), tumor-specific growth factor, are available as supplements to AFP in the detection of HCC. AFP mRNA has been shown to correlate with the metastasis and recurrence of HCC, and it may be the most useful biomarker for prognosis.

Des-gamma-carboxy prothrombin (DCP) is more sensitive and specific in diagnosing HCC when compared with AFP. DCP has a better diagnostic value than AFP in differentiating HCC from nonmalignant chronic liver disease. DCP has not only has a stronger correlation with HCC than AFP in tumor size but also more effectiveness than AFP in detecting small size of HCC. Biomarkers, such as gamma-glutamyl transferase mRNA, vascular endothelial growth factor, and interleukin-8, could also be used as available prognostic indicators, and the simultaneous determination of AFP and these markers may detect the recurrence of HCC at its earlier period. Serum RCP levels are significantly elevated in HCC also and could potentially serve as a biomarker for HCC.

A chromatin-remodeling enzyme, ALC1 (Amplified in Liver Cancer 1), also known as CHD1L, which interacts with poly(ADP-ribose) and catalyzes PARP1-stimulated nucleosome sliding, has been identified (Ahel et al. 2009). It is defined as a DNA damage-response protein, whose role in this process is sustained by its association with known DNA repair factors and its rapid poly(ADP-ribose)-dependent recruitment to DNA damage sites. Depletion or overexpression of ALC1 results in sensitivity to DNA-damaging agents. Collectively, these results provide new

insights into the mechanisms by which poly(ADP-ribose) regulates DNA repair. ALC1 is found in excessive amounts in half of liver cancers and can be used as a biomarker to pinpoint when liver cells start to become cancerous.

Several studies have shown that the blood levels of Golgi Protein-73 (GP73), a type II Golgi-localized integral membrane protein, are consistently higher in patients with primary liver cancer than in healthy individuals. In addition, levels are not significantly higher in patients with diseases other than liver disease. GP73 is a biomarker of HCC (Fimmel and Wright 2009). It is currently being tested in clinical trials and several medical diagnostic companies are developing automated serum tests for GP73 that could be performed in routine hospital laboratories. As a new diagnostic biomarker of PHC, GP73 protein in serum was highly sensitive and specific. Another study has shown that the combined detection of GP73 and AFP in serum effectively improves the diagnosis of HCC (Shi et al. 2011).

Biomarkers Indicating Lower Risk of HCC in Coffee Drinkers

A statistically significant attenuation (75%) of primary HCC risk among coffee drinkers (≥4 cups/day) has been confirmed for the inflammatory biomarker IL-6 and for the biomarkers of hepatocellular injury glutamate dehydrogenase, alanine aminotransferase, aspartate aminotransferase (AST), and γ-glutamyltransferase (GGT), and total bilirubin, which-in combination-attenuated the regression coefficients by 72% (Aleksandrova et al. 2015). Of the investigated biomarkers, IL-6, AST, and GGT produced the highest change in the regression coefficients: 40%, 56%, and 60%, respectively.

Metabonomic Profiles Discriminate HCC from Liver Cirrhosis

Biomarkers that discriminate HCC from LC are important but are limited. An ultra-performance LC-MS (UPLC-MS)-based metabonomics approach was used to characterize serum profiles from HCC, liver cirrhosis), and healthy subjects; the accuracy of UPLC-MS profiles and AFP) levels were compared for their use in HCC diagnosis (Wang et al. 2012). By multivariate data and receiver operating characteristic curves analysis, metabolic profiles were capable of discriminating not only patients from the controls but also HCC from LC with 100% sensitivity and specificity. Thirteen potential biomarkers were identified and suggested that there were significant disturbances of key metabolic pathways, such as organic acids, phospholipids, fatty acids, bile acids, and gut flora metabolism, in HCC patients. Canavaninosuccinate was first identified as a metabolite that exhibited a significant decrease in LC and an increase in HCC. In addition, glycochenodeoxycholic acid was suggested to be an important indicator for HCC diagnosis and disease prognosis. UPLC-MS signatures, alone or in combination with AFP levels, could be an efficient and convenient tool for early diagnosis and screening of HCC in high-risk populations.

Urinary Biomarkers of HCC

Urine is one of the most desirable body fluids for biomarker discovery as it can be easily obtained in large quantities and specimens are stable as compared with other body fluids. Protein expression profiles in urine from HCC patients and normal controls have been studied by shotgun proteomics using nanoLC-MS/MS and stable isotope dimethyl labeling to identify potential protein biomarkers for early diagnosis of HCC (Huang et al. 2015). Further integrated approach using proteomic, genomic and transcriptomic analysis identified that S100A9 and GRN were co-amplified and co-expressed in HCC and urine samples. In addition, the amplifications of S100A9 or GRN were found to be associated with poor survival in HCC patients, and their co-amplification also indicated worse overall survival than individual ones. These results suggest that urinary S100A9 and GRN as potential combinatorial biomarkers can be applied for early diagnosis of HCC.

Lung Cancer Biomarkers

Lung cancer is the leading cause of cancer-related death in Western nations. More than 300 million people die of this disease annually. In the US alone, 170,000 new cases of lung cancer are reported each year. Lung cancer is broadly divided into two types. Small-cell lung cancer (SCLC) accounts for approximately 80% of the all lung cancers and has a potential for cure by surgical resection. Non-small-cell lung cancer (NSCLC), an epithelial tumor, comprises about 20% of all lung cancers has a highly aggressive clinical course with tendency for early widespread metastases. Most of these are NSCLC and the overall prognosis once diagnosed is dismal. The only reasonable chance of cure is surgical resection for early stage tumors. However, most patients with early lung cancer are asymptomatic. Symptoms usually develop after the tumors become invasive or disseminated and curative resection is infeasible. Consequently, researchers have been working to find novel non-invasive or semi-invasive methods of identifying individuals who harbor progressive precancerous lesions. If detected early, these lesions might be treated with a chemopreventive agent to impede progress to invasive carcinoma.

Currently sputum cytology is considered to be the gold standard to assess the presence of malignant cells. Molecular biomarkers need to be validated before they are used in early clinical trials. Biomarkers for lung cancer are primarily involved in one of three major pathways: cell cycle regulation, apoptosis, and angiogenesis. Although no single biomarker has yet been shown to be perfect in predicting patient outcome, a profile based on the best of these biomarkers may prove useful in directing patient therapy. Various biomarkers for lung cancer are listed in Table 13.10.

Table 13.10 Biomarkers of lung cancer

Detection of cancer cells in sputum
Biomarkers in blood
Direct detection of circulating tumor cells in the blood
Tumor-derived DNA and RNA markers in blood
Biomarkers in exhaled breath:
miRNA biomarkers in lung cancer
Protein biomarkers
Antibodies: annexins I and II
Apolipoprotein C-I
Haptoglobin alpha-1 chain
S100A4
Serum protein biomarkers
Serum tNOX (tumor-associated NOX)
Gene expression profiling for biomarkers of lung cancer
Alterations of chromosomes: 3p, 5q, 9p
Genes: Rb, C-myc, C-mos, hTERT, K-ras, BRCA1, caveolin-1 (CAV1) and caveolin-2 (CAV2)
Proteins: p16, p53, hnRNP A2/B1, MCM2, EGFR, erbB-2, erbB-3, erbB-4, cyclophilin A (CyPA), CYPHRA211
Airway epithelial cell gene expression
miRNA expression in bronchial epithelium of smokers
Biomarkers closely associated with neuroendocrine differentiation in NSCLC
Pro-gastrin-releasing peptide (ProGRP)
Neuron-specific enolase (NSE)
Chromogranin A (CGA)
Biomarkers of inflammation
Cytokines: IL-6
C-reactive protein (CRP)
Methylation biomarkers
Nucleosomes in serum
Vascular endothelial cell growth factor

Autoantibodies as Biomarkers in Lung Cancer

Immune response manifested by annexins I and II autoantibodies occurs commonly in lung cancer and is associated with high circulating levels of IL-6 – an inflammatory cytokine. A proteomic approach using 2D PAGE, followed by Western blot analysis in which individual sera were tested for primary antibodies has led to the discovery of antiannexins I and/or II in sera from patients with lung cancer. Biomarkers have been detected in 90% of cases of lung adenocarcinoma. The CARET (Carotene and Retinol Efficacy Trial) feasibility study showed that anti-annexin antibodies could be detected in serum samples collected a year prior to clinical diagnosis of lung cancer.

Researchers at the University of Kentucky have described a fluorescent protein microarray to identify and measure multiple NSCLC-associated antibodies and showed how simultaneous measurements can be combined into a single diagnostic assay. Measurements of the five most predictive phage proteins were combined in a logistic regression model that achieved 90% sensitivity and 95% specificity in prediction of patient samples, whereas leave-one-out statistical analysis achieved 88.9% diagnostic accuracy among all 81 samples. In testing this marker set with samples from the Mayo Clinic Lung Screening Trial, the authors correctly predicted six of six prevalence cancers, 32 of 40 cancers from samples drawn 1 to 5 years before radiographic detection on incidence screening, and 49 of 56 risk-matched controls. These data indicate that antibody profiling is a promising approach that could achieve high diagnostic accuracy for NSCLC. These were licensed by 20/20 GeneSystems for development into a test for early detection of lung cancer using a combined panel of 4 biomarkers. This test can find the most common types of lung cancer and can identify risk at an early stage when intervention and treatment can improve survival chances. The test reports results using a simple risk score, generated from the panel of biomarkers, to give physicians insight on the likelihood that the patient has lung cancer. The reported result is a single numeric score with a cutoff showing a High/Low risk, relative to other persons with similar age and smoking history.

Panels of autoantibody serum assays in development by OncImmune Ltd. are unique in that they are designed to detect the first changes in proteins when cancer is developing. The assays have been optimized to display the proper epitopes in the proper orientation to bind to autologous antibodies. EarlyCDT-Lung test (OncImmune Ltd) measures a panel of 7 autoantibodies to detect the presence of lung cancer.

Biomarkers Associated with Neuroendocrine Differentiation in NSCLC

Pro-gastrin-releasing peptide (ProGRP), along with Chromogranin A (CGA), and neuron-specific enolase (NSE) belongs to a group of immunohistochemical tissue biomarkers closely associated with neuroendocrine differentiation in NSCLC. Serum levels of these biomarkers can also be measured and are used in predicting response to chemotherapy and survival of patients with unresectable NSCLC. In NSCLC patients on chemotherapy, serum CGA and Pro-GRP have been reported to provide important information related to the prognosis for NSCLC patients before chemotherapy. While a high CGA before treatment is an unfavorable prognostic determinant, a high ProGRP confers a survival advantage. Thus, combined use of serum CGA, ProGRP and NSE may supply additional information for prognosis. Tumor marker serum levels have been correlated with histological type and tumor extension, with ProGRP being the most sensitive marker in SCLC, CEA in adenocarcinomas and CYFRA 21-1 in squamous tumors of the lung. The most sensitive combinations of tumor biomarkers are ProGRP and NSE in SCLC (88%), and CEA plus CYFRA 21-1 in NSCLC (82%). Thus ProGRP is the tumor biomarker of

choice in SCLC and NSE is a complementary tumor marker in this histological type. ProGRP is being developed commercially by a collaboration of Abbott Diagnostics and Advanced Life Science Institute.

Biomarkers of Chronic Inflammation in Lung Cancer

Chronic inflammation has been implicated in the development of airway dysplasia and lung cancer. C-reactive protein (CRP), a biomarker for inflammation in the blood, can help to identify individuals whose abnormal precancerous lesions will advance closer to invasive lung cancer. Plasma CRP, in concert with lung function and pack-years of smoking, appears to have excellent predictive powers in identifying participants with bronchial dyplastic lesions whose lesions progress to more advanced stages of dysplasia. The odds of developing progressive disease are 9.6-fold higher in the group with CRP >0.5 mg per liter compared with the group less than this threshold.

Biomarkers for Predicting Sensitivity to Chemotherapy in Lung Cancer

Platinum-based chemotherapy is the standard treatment in advanced NSCLC and as adjuvant treatment in a substantial subgroup of patients with stage II-IIIA. Therefore, there is an interest in biomarkers for predicting sensitivity to platinum compounds. The expression of genes involved in DNA repair pathways, particularly genes involved in the nucleotide excision repair, has an important role in predicting response to platinum-based chemotherapy. Therefore, patients with low DNA repair capacity should have a favorable response to gemcitabine plus carboplatin chemotherapy, whereas they could be resistant to docetaxel. However, cisplatin can induce ERCC1 (excision repair cross-complementing 1) mRNA levels, which might be a principal reason for short-lived response to platinum agents. Although several trials evaluated the level of expression of ERCC1, no consensus was reached regarding a method for evaluation. A study has used the 8F1 antibody to measure the level of expression of ERCC1 by IHC analysis in a validation set of samples obtained from patients in two independent phase III trials (Friboulet et al. 2013). Unique functional ERCC1 isoform was not specifically detected; therefore, its usefulness in guiding therapeutic decision making is limited. BRCA1 complexes are also central to DNA repair response, and the influence of RAP80 expression in conjunction with BRCA1 expression is now being investigated in phase III randomized clinical trials of customized chemotherapy.

In patients with inoperable SCLC, the efficacy of chemotherapy can be predicted early in the course of therapy by baseline values of serum nucleosomes as independent parameters. In prospectively collected sera of patients with recurrent NSCLC receiving second-line chemotherapy, the courses of nucleosomes, cytokeratin-19 fragments (CYFRA 21-1), CEA, neuron-specific enolase, and progastrin-releasing peptide were investigated and correlated with therapy response

(Holdenrieder et al. 2009). At high specificity for detection of progressive disease, most sensitive biomarkers were identified and included in a combination model. High levels and insufficient decreases of nucleosomes and CYFRA 21-1 during the first cycle of therapy indicated poor outcome. Combination of nucleosome concentrations and CYFRA 21-1 enabled the early detection of progressive disease. Nucleosomes and CYFRA 21-1 were shown to be valuable for the individual management of patients with recurrent NSCLC.

Platinum-based chemotherapy is a primary treatment for patients with advanced NSCLC. There is need for a convenient method is to identify the sensitivity of individual patient to platinum-based regimen. Genetic variants in DNA repair genes represent important determinants of drug efficacy. Xeroderma pigmentosum group A (XPA) codon23 and xeroderma pigmentosum group D (XPD) codon751 SNPs are involved in clinical response to platinum-based chemotherapy in advanced NSCLC patients. A study has confirmed that XPA A23G, a SNP in blood cells detected by 3D polyacrylamide gel-based DNA microarray method, might be a promising biomarker in predicting favorable prognosis of NSCLC patients and designing individualized treatments (Cheng et al. 2013).

Several other biomarkers of response of lung cancer to chemotherapy have been identified. There is need for clinical trials to prospectively validate the benefits of customizing chemotherapy for translation into an improvement in outcome of NSCLC patients.

Biomarkers for Prediction of Sensitivity to EGFR Inhibitors

Epidermal growth factor receptor (EGFR) Inhibitors have shown promising results in patients with advanced NSCLC who previously have failed on chemotherapy. A major problem is how to select the patients, who will benefit from treatment, and who will not. The predictive role of EGFR protein expression assessed by IHC is still debated. Specific EGFR gene mutations have been identified associated with response to gefitinib (Iressa®), but seem not to be associated with stable disease. Other biomarker studies are described, which are associated with sensitivity to EGFR inhibitors. Increased EGFR gene copy number based on FISH analysis is demonstrated to be a good predictive marker for response, stable disease, time to progression, and survival. However, EGFR mutation is a better predictor of clinical outcome in gefitinib-treated patients than the EGFR gene copy number. EGFR/FISH seems to be the best predictive iomarker for clinical benefit from EGFR inhibitors in NSCLC. Prospective large scale clinical studies must identify the most optimal paradigm for selection of patients.

Previously, tumor biopsies have been used for EGFR genotyping in NSCLC as it has been difficult to detect the low levels of specific mutations shed from the tumor into the blood against the high background of normal DNA. Testing DNA isolated from blood, rather than tumor tissue, would be better for predicting responses to gefitinib, erlotinib (Tarceva) and other cancer therapies. If EGFR mutations can be observed in serum DNA, this could serve as a noninvasive source of information on

the genotype of the original tumor cells as compared to direct sampling of the tumor and could influence treatment and the ability to predict patient response to gefitinib. Patients with EGFR mutations seem to have better outcomes with gefitinib (Iressa) treatment, in terms of progression-free survival, overall survival, and response, than those patients without EGFR mutations.

A phase I, dose-escalation, combination drug study of subjects with NSCLC receiving celecoxib and erlotinib (Tarceva) has identified proteins that may eventually act as biomarkers guiding advanced lung cancer treatment (Krysan et al. 2008). All of these advanced lung cancer patients had previously received conventional treatments without success. About half of the patients had positive outcomes on the therapy defined as tumors that did not grow or that decreased in size by more than 30%. Use of ELISA to analyze patients' serum protein levels at various time points over 2 months revealed an intriguing link between treatment response and the levels of four proteins: soluble E-cadherin, a matrix metalloprotein (MMP), a tissue inhibitor of MMP, and CCL15. These proteins act downstream of COX-2, an enzyme causing inflammation that can hinder cancer treatment by vascularizing tumors and making them more resilient. COX-2, which is overexpressed in 80–85% of lung cancers also seems to hinder the effect of drugs such as erlotinib, which target EGFR on tumor cell surfaces. Combination therapy with the COX-2 inhibitor celecoxib is intended to restore tumor cell sensitivity and enhance erlotinib's efficacy, since only 15% of patients respond to erlotinib alone. In this study, the patients who showed positive outcomes after eight weeks also had lower sEC, TIMP-1, and CCL15 serum levels, while patients with low MMP-9 levels before treatment showed the best treatment results. The latter result indicates the usefulness of MMP-9 as a treatment biomarker for determining the appropriateness of the new combination drug treatment. A larger, phase II, multi-center study will verify their preliminary association between tumor size and protein levels during combination drug treatment.

In 2008, the NIH started a 4-year phase III study, Marker Validation for Erlotinib in Lung Cancer (MARVEL), to validate if choosing patients with NSCLC for Erlotinib based on the presence or absence of EGFR activation has a meaningful benefit over the standard chemotherapy the patients received. The trial (NCT00738881) was terminated.

CTCs as Biomarkers of Lung Cancer

In lung cancer, the biological assessment of CTCs therapeutic target biomarkers, such as mutations of EGFR, could be a valid alternative to its determination in tumor samples. EGFR mutation analysis in CTC was shown to be concordant with data from tumoral samples (Maheswaran and Haber 2010). CTCs characteristics in lung cancer could help to stratify the patients and possibly drive future therapeutic strategies. CTC numbers correlate with prognosis in both early and advanced lung cancer. The availability of this noninvasive virtual biopsy could solve the problem of the increasing need of lung cancer biological samples for molecular studies.

This would help avoid the use of invasive procedures in order to obtain cytological specimens or small biopsies (Franco et al. 2012).

Gene Expression Profiling for Biomarkers of Lung Cancer

The most common genetic changes associated with lung cancer involve abnormalities of the genes and the proteins expressed by them, which regulate the cell cycle. Molecular networking of P53 and P16 tumor suppressor genes and K-Ras oncogene exerts a crucial impact on cell cycle regulation and appears to be of major clinical significance for lung cancer evaluation. P53, P16 and K-Ras evaluations have been used in lung cancer with particular focus on biological and clinical implications, as well as on new molecular approaches to the study of these genes.

Sixteen genes that correlated with survival among patients with NSCLC were identified by analyzing microarray data and risk scores (DUSP6, MMD, STAT1, ERBB3, and LCK) were selected for RT-PCR and decision-tree analysis (Chen et al. 2007). The five-gene signature is closely associated with relapse-free and overall survival among patients with NSCLC. BRCA1 and Xeroderma pigmentosum group G (XPG) are independent prognostic factors for both median survival and disease-free survival. High BRCA1 mRNA expression confers poor prognosis in early NSCLC, and the combination of high BRCA1 and low XPG expression still further increases the risk of shorter survival (Bartolucci et al. 2009). These findings can help optimize the customization of adjuvant chemotherapy.

A subset of 11 genes has been identified as a prognostic gene-expression signature and validated in multiple independent NSCLC microarray datasets (Navab et al. 2011). Functional annotation using protein-protein interaction analyses of these and published cancer gene-expression changes revealed prominent involvement of the focal adhesion and MAPK signaling pathways. Fourteen of the 46 genes were also differentially expressed in LCM primary tumor stroma compared with the matched normal lung. Six of these 14 genes could be induced by TGF-β1 in normal fibroblasts. These results establish the prognostic impact of changes in gene-expression of carcinoma-associated fibroblasts in NSCLC patients.

Because cigarette smoke injures the airway, efforts have been made to determine if gene expression in histologically normal large-airway epithelial cells obtained at bronchoscopy from smokers with suspicion of lung cancer could be used as a lung cancer biomarker. Using a training set and gene-expression profiles from Affymetrix HG-U133A microarrays, an 80-gene biomarker was identified that distinguishes smokers with and without lung cancer (Spira et al. 2007). This biomarker had ~90% sensitivity for stage 1 cancer across all subjects. Combining cytopathology of lower airway cells obtained at bronchoscopy with the biomarker yielded 95% sensitivity and a 95% negative predictive value. These findings indicate that gene expression in cytologically normal large-airway epithelial cells can serve as a lung cancer biomarker, potentially owing to a cancer-specific airway-wide response to cigarette smoke.

Methylation Biomarkers of Lung Cancer

A DNA analysis technique called methylation profiling identifies molecular biomarkers in early lung cancer. Biomarkers of lung cancer have been identified by Epigenomics AG with its differential methylation hybridization technology and have been extensively validated on tissue samples before being tested on blood plasma. A large clinical trial has confirmed that a two-biomarker panel correctly identified two-thirds of all lung cancers in blood plasma at a false positive rate of 12% (88% specificity). Most of the blood samples used in the study were obtained from patients with early stage I and II cancer. Sensitivity in stage II lung cancer patients reached 73%. Patients with early stage cancer are significantly underdiagnosed in the current diagnostic practice for lung cancer but could benefit most from early therapeutic intervention.

Despite optimal and early surgical treatment of NSCLC, many patients die of recurrence. This led to investigation of association between gene methylation and recurrence of the tumor. In a multivariate model, the following were associated with tumor recurrence, independently of NSCLC stage, age, sex, race, smoking history, and histologic characteristics of the tumor (Brock et al. 2008):

1. Promoter methylation of the cyclin-dependent kinase inhibitor 2A gene p16;
2. H-cadherin gene CDH13;
3. Ras association domain family 1 gene RASSF1A; and
4. Adenomatous polyposis coli gene APC in tumors and in histologically tumor-negative lymph nodes.

It was concluded that methylation of the promoter region of the four genes in patients with stage I NSCLC treated with curative intent by means of surgery is associated with early recurrence.

miRNA Biomarkers in Lung Cancer

Let-7, a natural and separately transcribed miRNA maps to a chromosomal region associated with lung cancer as a regulator of Ras expression. Let-7 expression is lower in lung tumors than in normal lung tissue, while RAS protein is significantly higher in lung tumors, providing a possible mechanism for let-7 in cancer. The let-7 miRNA regulates Ras by binding to the message for Ras and likely inhibits translation of the Ras protein rather than revertion of a mutated Ras to normal. The LCS6 variant allele in a KRAS miRANA complementary site is significantly associated with increased risk for NSCLC among moderate smokers and links let-7 miRNAs to lung cancer susceptibility (Chin et al. 2008). These findings opens up the possibility that gene therapy with let-7 may alleviate or slow down lung cancer.

Studying current and non-smokers, researchers from Boston University School of Medicine have examined whole-genome miRNA and mRNA expression in bronchial airway epithelial brushings obtained at bronchoscopy and found 28 miRNAs to be differentially expressed in the majority of smokers. In addition, they showed

that modulating the expression of one of these miRNAs, mir-218, was sufficient to alter the expression of a subset of the mRNAs that are both predicted targets of this miRNA and altered by smoking in vivo. These studies suggest that smoking-dependent changes in miRNA expression levels mediate some of the smoking induced gene expression changes in airway epithelium and that miRNAs therefore play a role in the host response to environmental exposures and may contribute to the pathogenesis of smoking-related lung cancer. It is hoped that miRNA profiles obtained from these cells may serve as relatively noninvasive biomarkers for smoking-related lung diseases.

A study has explored miRNA expression profiles of lung tumors, normal lung tissues and plasma samples from cases with variable prognosis identified in a completed spiral-CT screening trial with extensive follow-up (Boeri et al. 2011). miRNA expression patterns significantly distinguished: (i) tumors from normal lung tissues; (ii) tumor histology and growth rate; (iii) clinical outcome; and (iv) year of lung cancer CT detection. Thus miRNAs play a role in lung tissues and plasma as molecular predictors of lung cancer development and aggressiveness and have theoretical and clinical implication for lung cancer management. The diagnostic ability of different miRNA biomarkers varies among reported studies. A systematic review has used diagnostic values analysis to summarize the overall test performance of miRNAs (Huang et al. 2014). The results indicate that miRNAs in body fluids accurately identify NSCLC and are useful for diagnosis.

Noninvasive Detection of Lung Cancer Using Exhaled Breath

Analysis of exhaled breath is promising as a noninvasive diagnostic tool for diagnosis of lung cancer. Collection exhaled breath condensate (EBC) is a simple and noninvasive technique, which enables sampling of lower respiratory tract fluid. Proteomic analysis of EBC may be used as a tool for early detection of lung cancer; a review of of this approach along with discussion of benefits, pitfalls and possible future developments has been published (Eberini et al. 2008). Advances in proteomic technologies are expected to validate EBC proteins as biomarkers to enable early detection of lung cancer.

Another study has demonstrated the quantitative analysis of carbonyl volatile organic compounds (VOCs) and identification of VOC biomarkers of lung cancer in exhaled breath using unique silicon microreactor technology (Fu et al. 2014). The microreactor consists of thousands of micropillars coated with an ammonium aminooxy salt for capture of carbonyl VOCs in exhaled breath by means of oximation reactions. Captured aminooxy-VOC adducts are analyzed by nanoelectrospray Fourier transform-ion cyclotron resonance MS. The concentrations of 2-butanone, 2-hydroxyacetaldehyde, 3-hydroxy-2-butanone, and 4-hydroxyhexenal in the exhaled breath of lung cancer patients were significantly higher than in the exhaled breath of healthy controls and patients with benign pulmonary nodules. The concentration of 2-butanone in exhaled breath of patients with stages II to IV of NSCLC was significantly higher than in exhaled breath of patients with stage I. The carbonyl

VOC profile in exhaled breath determined using this new silicon microreactor technology enables noninvasive detection of lung cancer.

Serum Protein Biomarkers of Lung Cancer

There is an enormous unmet medical need related to the diagnosis of lung cancer in the earliest stages when it is most treatable but no approved blood test for lung cancer is yet available. Serum biomarkers that could aid clinicians in making case management decisions about lung cancer would be extremely useful. Two proteomic platforms and literature search have enabled selection of candidate serum biomarkers for the diagnosis of lung cancer (Patz et al. 2007). Classification and Regression Tree (CART) analysis was used to select a panel of four serum protein biomarkers for prediction of lung cancer in patients: (1) carcinoembryonic antigen; (2) retinol binding protein; (3) alpha1-antitrypsin; and (4) squamous cell carcinoma antigen. These were collectively found to correctly classify the majority of lung cancer and control patients in the training set (sensitivity 89.3% and specificity 84.7%). These biomarkers also accurately classified patients in the independent validation set (sensitivity 77.8% and specificity 75.4%). Approximately 90% of patients who fell into any one of three groupings in the CART analysis had lung cancer. Thus the panel of four serum proteins is valuable in the diagnosis of lung cancer. The data may be useful for treating patients with an indeterminate pulmonary lesion, and potentially in predicting individuals at high risk for lung cancer. Laboratory Corporation of America plans to market the serum protein assay, which could serve as a useful complement to imaging studies such as CT scan to differentiate cancers from benign nodules.

An efficient strategy, consisting of SELDI-TOF-MS analysis, HPLC purification, MALDI-TOF-MS trace and LC-MS/MS identification is useful for detection of protein biomarkers. Apolipoprotein C-I, haptoglobin alpha-1 chain, and S100A4 have been identified as potential proteomic biomarkers of NSCLC but further studies with larger sample sizes will be needed to validify these (Yang et al. 2009a).

Each year, millions of pulmonary nodules are discovered by CT and subsequently biopsied. Because most of these nodules are benign, many patients undergo unnecessary and costly invasive procedures. A 13-protein blood-based classifier helps to differentiate malignant and benign nodules with high confidence, thereby providing a diagnostic tool to avoid invasive biopsy on benign nodules (Li et al. 2013). Using a systems biology strategy, the authors of the study identified several protein candidates and developed a multiple reaction monitoring (MRM) assay for each, which was applied in a discovery study on plasma samples from patients with benign and stage IA lung cancer matched for nodule size, age, gender, and clinical site, producing a 13-protein classifier. The classifier (set of biomarkers) was validated on an independent set of plasma samples, exhibiting a negative predictive value (NPV) of 90%. Validation performance on samples from a nondiscovery clinical site showed an NPV of 94%, indicating the general effectiveness of the classifier. A pathway analysis demonstrated that the classifier proteins are likely modulated by a few transcription regulators (NF2L2, AHR, MYC, and FOS) that are associated with lung cancer, lung inflammation, and oxidative stress networks. The classifier

score was independent of patient nodule size, smoking history, and age, which are risk factors used for clinical management of pulmonary nodules. This test is commercialized as Xpresys Lung (Integrated Diagnostics) to simultaneously measure multiple circulating proteins associated with lung cancer by the most advanced mass spectrometry. This test is a complementary tool in diagnosis of lung cancer.

Human primary lung adenocarcinoma tumors have been analyzed using global MS to elucidate the biological mechanisms behind relapse after surgery (Pernemalm et al. 2013). In total, >3000 proteins were identified with high confidence and supervised multivariate analysis was used to select 132 proteins separating the prognostic groups. Based on in-depth bioinformatics analysis, the authors hypothesized that the tumors with poor prognosis had a higher glycolytic activity and HIF activation. By measuring the bioenergetic cellular index of the tumors, they could detect a higher dependency of glycolysis among the tumors with poor prognosis. Further, they could also detect an up-regulation of HIF1α mRNA expression in tumors with early relapse. Finally, they selected three proteins that were upregulated in the poor prognosis group (cathepsin D, ENO1, and VDAC1) to confirm that the proteins indeed originated from the tumor and not from a stromal or inflammatory component. Overall, these findings show how in-depth analysis of clinical material can lead to an increased understanding of the molecular mechanisms underlying tumor progression. This study shows a functional coupling between high glycolytic activity and postsurgical relapse of adenocarcinoma of the lung. Protein level changes detected in this study could serve as starting point for discovery of predictive biomarkers for metabolic treatment options in lung cancer.

tNOX as Biomarker of Lung Cancer

tNOX (tumor-associated NOX) is a member of a family of proteins that are involved in cell growth. Normal cells express the NOX enzyme only when they are dividing in response to growth hormone signals. In contrast, cancer cells have gained the ability to express NOX activity at all times. This overactive form of NOX, known as tNOX is vital for the growth of cancer cells, because drugs that inhibit tNOX activity also block tumor cell growth in culture. Serum tNOX test is being developed as a screening tool for the early detection of lung cancer. Those who test positive can be followed up with a medical examination and further tests, ostensibly including high resolution CT. This test is structured with the antibody for lung cancer in one form or another and is a specific diagnosis that also distinguishes between NSCLC and SCLC.

Tumor-Derived DNA and RNA Markers in Blood

PCR enables the detection and quantification of extremely small amounts of tumor-derived nucleic acids. This has led to an increased knowledge of the molecular pathogenesis of lung cancer and a basis for the use of DNA and RNA markers in blood for early cancer detection, diagnostics, and follow-up. Common genetic

alterations in lung carcinogenesis are already well known. Several clinical studies have evaluated the role of DNA and RNA aberrations in the blood of lung cancer patients and overall plasma/serum abnormalities were found in a high percentage of patients with lung cancer as compared with healthy controls. The analysis of circulating DNA or RNA in plasma is a promising non-invasive diagnostic tool, requiring only a limited blood sample. Its wide applicability and potential importance will possibly lead to increasing clinical impact in the near future. However, large prospective clinical studies are needed to validate and standardize any tests for DNA or RNA alteration in plasma or serum of high risk individuals or patients with established lung cancer.

Volatile Organic Compounds in the Exhaled Breath

The pattern of volatile organic compounds in the exhaled breath of lung cancer patients may be unique. Novel sensor systems that detect patterns of volatiles have been developed. One of these sensor systems, a colorimetric sensor array, has 36 spots composed of different chemically sensitive compounds impregnated on a disposable cartridge (Mazzone et al. 2007). The colors of these spots change based on the chemicals they come in contact with. The color changes that occur for each individual are converted into a numerical vector. The vectors were analyzed statistically, using a random forests technique, to determine if lung cancer could be predicted from the sensor responses. A prediction model was developed using observations from 70% of the subjects with various lung diseases including cancer. This model was able to predict the presence of lung cancer in the remaining 30% of the subjects with a sensitivity of 73.3% and specificity of 72.4%. Thus the unique chemical signature of the breath of lung cancer patients can be detected with moderate accuracy by a colorimetric sensor array.

Malignant Pleural Mesothelioma

Malignant pleural mesothelioma (MPM) is a highly aggressive neoplasm of lung pleura with poor prognosis. Exposure to asbestos fibers is the primary cause of MPM with as many as 80% of the patients having been exposed to asbestos. Recent age standardization rates for mesothelioma in men in Australia, is estimated to be 6 per 100,000. Although MPM remains a relatively uncommon malignancy, it continues to represent an important cause of mortality in numerous areas worldwide; e.g. England, Wales, continental Europe, and Australia.

MPM remains difficult to detect early and treat effectively. Novel proteomic technologies can be utilized to discover changes in expression of pleural proteins that might have diagnostic value. SELDI-TOF and MS can be used to detect protein profiles in pleural effusions that could identify MPM. Mesomark™ (Fujirebio Diagnostics Inc) is an ELISA for the quantitative measurement of soluble mesothelin

related peptides (SMRP) in human serum that are related to the mesothelin/megakaryocyte potentiating factor (MPF) family of proteins and recognized by the MAb OV569. The reactivity of OV569 is low for normal human tissues except for the mesothelium. In a study on pleural effusions from patients with confirmed MPM and from patients with effusions due to other causes, various commercially available immunoassays were used to detect human epididymis protein 4 (HE4), osteopontin (OPN), SMRP, and the cytokeratin 19 fragment (CYFRA 21-1) and peak intensity data obtained by SELDI-TOF were subjected to classification algorithms in order to identify potential classifier peaks (Hegmans et al. 2009). A protein peak at m/z 6614 was characterized as apolipoprotein (Apo) CI. In this setting, the sensitivity and specificity of the potential biomarker, Apo CI, was 76% and 69%, respectively, thereby outperforming OPN, HE4, and CYFRA 21-1. This study validates the use of SMRP (as measured by Mesomark™) as a diagnostic biomarker for pleural mesothelioma and furthermore suggests that Apo CI levels could be used in the future to discriminate MPM from other causes of pleural exudate.

Melanoma Biomarkers

Cutaneous malignant melanoma remains the leading cause of skin cancer death in industrialized countries. Melanoma is diagnosed in more than 50,000 new patients in the US annually. Melanoma progression is well defined in its clinical and histopathological aspects (Breslow's index, tumor size, ulceration, or vascular invasion), which also give hints to prognosis of the patient. Use of molecular biomarkers should therefore give additional information which cannot be determined by routine histopathology. There is a critical unmet need for new predictive and prognostic biomarkers for melanoma, particularly ones that can identify those tumors that are likely to result in progression (metastasis) and death. A classification of biomarkers of melanoma is shown in Table 13.11.

Several molecules influencing invasiveness and metastatic dissemination of melanoma have been identified. Expression of these molecules has been studied in primary melanoma and correlated with prognosis. Moreover, several tumor suppressors and oncogenes have been shown to be involved in melanoma pathogenesis, including CDKN2A, PTEN, TP53, RAS and MYC, but have not been related to melanoma subtypes or validated as prognostic markers. In the past, an increase in the number of positive tumor cells for Ki67, cyclin A, cyclin D, MMP-2, integrins beta1 and beta3 or osteonectin, as well as the decrease in p16, p27, and Melan A were considered as factors of poor prognosis in melanoma. However, only a small subset of these proteins has a prognostic value independent of tumor thickness. Development of high-throughput technologies fornanalyzing global molecular profiles of cancer is discovering previously unknown candidate genes involved in melanoma, such as Wnt-5A and B-raf.

YKL-40 is a growth factor for connective tissue cells and stimulates migration of endothelial cells. Cancer cells, macrophages, and neutrophils secrete YKL-40.

Table 13.11 Classification of biomarkers of melanoma

Genes
B-raf
Oncogenes: CDKN2A, MYC, RAS
Tumor suppressors: PTEN, TP53
Wnt-5A
Protein biomarkers
Cell cycle associated proteins: Cyclin A, B, C, D
Matrix metalloproteinases (MMP)-1 and −9
Melan A
Melanoma-inhibiting activity (MIA)
Melastatin
p16
p27
S100B
Methylation biomarkers
MicroRNA biomarkers
Suppressed natural killer (NK) cell function
NKG2D
CD158a
CD158b
Serum biomarkers
Lactic dehydrogenase (LDH)
Melanoma cell adhesion molecules: soluble intracellular adhesion molecule 1 (sICAM-1)
Melanoma-inhibiting activity (MIA)
Melanocyte lineage/differentiation antigens: S100B
TA90-immune complex (TA90IC)
YKL-40
Imaging biomarkers
DCE-MRI

Elevated serum YKL-40 is an independent prognostic factor for poor survival in patients with metastatic melanoma.

The first unbiased systematic effort to determine methylation biomarkers in melanoma by mapping chemical modifications of DNA in the melanoma genome has revealed several novel genes regulated by promoter methylation that were not described in cancer cells before (Koga et al. 2009). Discovery of new methylation biomarkers will help develop more effective treatment strategies to fight this disease.

Currently, no biomarker to improve risk stratification is part of recommended clinical practice. Although numerous biomarkers candidates have been identified, their relevance to melanoma progression, clinical outcome and the selection of optimal

treatment strategies still needs to be established. A more accurate, therapeutically predictive classification of human melanomas and selection of patient populations that would profit from therapeutic interventions are among the major challenges expected to be addressed in the future. Biomarker identification and validation will provide a rapidly changing molecular view of melanoma, a strategy that is necessary for developing truly stratified or personalized prevention or management.

Nasopharyngeal Carcinoma Biomarkers

Nasopharyngeal carcinoma (NPC) is a rare malignancy in most part of the world and it is one of the most poorly understood and commonly misdiagnosed diseases. It is highly prevalent in southern Asia where the disease prevalence is ~100-fold higher compared with other populations not at risk. As one of the most common cancers among Chinese or Asian ancestry, it poses one of the serious health problems in southern China where an annual incidence of >20 cases per 100,000 is reported (Cho 2007). Men are twice as likely to develop NPC as women. The incidence increases from ages 20 to 50. The cause of NPC is a complex interaction of genetic, viral (Epstein-Barr virus), environmental and dietary factors. Diagnosis of NPC at an early disease stage is important for successful treatment and improving the outcome of patients. Biomarkers of nasopharyngeal carcinoma and potential applications are shown in Table 13.12.

Proteomic Biomarkers of Nasopharyngeal Cancer

The use of serum protein profiles and a classification tree algorithm have been explored to distinguish NPC from noncancer control. SELDI-TOF-MS serum protein profiles can discriminate NPC from noncancer. The combination of serum protein profiles with an Epstein Barr virus antibody serology test can further improve the accuracy of NPC screening.

Among the biomarkers under investigation, Bmi-1 oncoprotein regulates proliferation and oncogenesis in human cells, is promising. Its overexpression leads to senescence bypass in human fibroblasts and immortalization of human mammary epithelial cells. Bmi-1 is overexpressed in NPCs, whereas there is no detectable expression of Bmi-1 in noncancerous nasopharyngeal epithelium cells (NPEC). Moreover, high Bmi-1 expression positively correlates with poor prognosis of NPC patients. Overexpression of Bmi-1 leads to bypass of senescence and immortalization of NPECs, which normally express p16(INK4a) and exhibit finite replicative life span. Overexpression of Bmi-1 in NPECs leads to the induction of human telomerase reverse transcriptase activity and reduction of p16(INK4a) expression. These findings indicate that Bmi-1 plays an important role in the development and progression of BMC, and is a valuable biomarker for assessing the prognosis of NPC.

Table 13.12 Biomarkers of nasopharyngeal carcinoma and potential applications

Biomarkers discovered by use of proteomic technologies
Annexin A2
Basic transcription factor 3: signaling target
Ceruloplasmin: enhanced levels are normalized after positive response to therapy
Fibronectin: diagnosis
Heat shock protein 27: signaling target
Inter-α-trypsin inhibitor precursor: diagnosis
Mac-2 binding protein: diagnosis
Plasminogen activator inhibitor 1: diagnosis
Platelet factor-4: monitoring of response to treatment
Porin: signaling target
Serum amyloid: ProteinChip analysis to monitor relapse of NPC
Stathmin: signaling target
miRNA biomarkers of nasopharyngeal cancer
Tumor suppressor genes as molecular biomarkers of targeted therapies
THY1: associated with lymph node metastatic potential of NPC
BLU/ZMYND10: downregulated in NPC
GADD45G: its response to environmental stresses is disrupted epigenetically in NPC
14-3-3σ gene product: up-regulated by p53 in response to DNA damage and downregulated in NPC.
Gene expression biomarkers as targets for therapy
Death-associated protein kinase (DAPK): Loss of expression is associated with promoter region methylation in NPC. Potential reactivation by 5-Aza-2′-deoxycytidine.
EGFR: silencing by RNAi reduces the proliferation of NPC cells
Survivin: role in resisting apoptosis in NPC was confirmed by RNAi
Molecular biomarkers for prognosis and monitoring response to treatment
Endothelin-1: pretreatment plasma levels for predicting posttreatment failure in advanced NPC.
Epstein-Barr virus (EBV): antibodies and EBV DNA are useful for the early detection, monitoring and prognosis of NPC.
Heparanase: overexpression is inversely correlated with survival of NPC patients
Tiam1: overexpression correlates with invasion and metastasis of NPC.
IL-8 receptor A: overexpression in tumor cells indicates poor prognosis
VEGF: overexpression is associated with poor prognosis of NPC

miRNA Biomarkers of Nasopharyngeal Carcinoma

Use of highly sensitive microarray-based procedures has enabled the identification of eight miRNAs showing robust differential expression between laser-capture-microdissected nasopharyngeal carcinomas (NPCs) and normal healthy nasopharyngeal epithelial samples (SenGupta et al. 2008). In particular, miRNA mir-29c was expressed at one-fifth the levels in tumors as in normal epithelium. In NPC

tumors, the lower mir-29c levels correlated with higher levels of multiple mRNAs whose 3′ UTRs can bind mir-29c at target sequences conserved across many vertebrates. In cultured cells, introduction of mir-29c down-regulated these genes at the level of mRNA and inhibits expression of luciferase encoded by vectors having the 3′ UTRs of these genes. Most of the mir-29c-targeted genes identified encode extracellular matrix proteins, including multiple collagens and laminin γ1, which are associated with tumor cell invasiveness and metastatic potential, prominent characteristics of NPC. Thus, we eight miRNAs are differentially expressed in NPC and are involved in regulating genes involved in metastasis.

NPC is associated with Epstein-Barr virus (EBV) infection, which was the first human virus found to encode miRNAs. EBV-encoded protein LMP1 is believed to be a key factor in NPC development. EBV miRNAs plays a role in regulating LMP1 downstream signaling to promote cancer development (Lo et al. 2007).

Oral Cancer Biomarkers

Oral cancer is a major global threat to public health with 300,000 new cases diagnosed worldwide on an annual basis. Oral cancer development is a tobacco-related multistep and multifocal process involving field carcinogenesis and intraepithelial clonal spread. The morbidity and mortality rates of this devastating disease have not improved in decades. Biomarkers of genomic instability, such as aneuploidy and allelic imbalance, can accurately measure the cancer risk of oral premalignant lesions or intraepithelial neoplasia (IEN). Retinoid-oral IEN studies (e.g., retinoid acid receptor-beta, p53, genetic instability, loss of heterozygosity, and cyclin D1) have advanced the overall understanding of the biology of intraepithelial carcinogenesis and preventive agent molecular mechanisms and targets, important advances for monitoring preventive interventions, assessing cancer risk, and pharmacogenomics. However, there is a lack of plasma biomarker for detecting oral cancer.

Both IL-8 and IL-1β are expressed at significantly higher levels in oral squamous cell carcinoma subjects than in the matched healthy control subjects. Luminex xMAP single-plex and multiplex assays are as effective as ELISA assays for quantification of biomarker proteins in saliva (Arellano-Garcia et al. 2008).

Ovarian Cancer Biomarkers

Ovarian cancer is the fourth leading cause of cancer deaths among women in the US, despite its relatively low incidence of 50 per 100,000. On average, US women have a 2% risk of developing ovarian cancer by age 70. Risk factors for ovarian cancer include previous breast cancer, family history of breast or ovarian cancer and hereditary non-polyposis colorectal cancer. Thus approximately 10 million women in the US are at risk for the development of ovarian cancer and should be tested for

it. Most women are diagnosed with ovarian cancer in late-stage disease and have a 5-year survival rate of less than 30% but these rates soar to over 90% if the disease is discovered when cancer still is localized to the ovaries.

Clinical diagnosis of ovarian tumors is difficult in the absence of physical symptoms as the ovaries are located deep in the abdomen. Unfortunately, current methods of diagnosis, such as transvaginal ultrasound, laparoscopy or PET scan are impractical for general testing as they are complex procedures and would pose a tremendous burden on the healthcare system. The need for an accurate yet simple test has prompted investigators to explore novel, rapid ways of detecting ovarian cancer. To be practical for testing the high-risk population, a rapid detection assay for ovarian cancer should have the possibility to be performed on an easily obtained specimen, such as a blood sample. An assay that requires a tissue biopsy, for example, would be ruled out as a convenient test. The assay must be robust, such that normal handling and transport of the specimen to the testing laboratory does not alter the analyte or biomarker being measured. The need for special sample processing not normally performed at the patient service center, such as flash-freezing or extraction of a particular molecular component could make it difficult to adapt the test for routine use. In the laboratory, the process for testing the sample should be high-throughput and automated. Several tests are in development for ovarian cancer. Biomarkers for ovarian cancer are listed in Table 13.13 and tests relevant to biomarkers are discussed in the following sections.

Table 13.13 Biomarkers of ovarian cancer

Serum CA125 (MUC16)
Serum lysophosphatidic acids
Serum protein biomarkers
Biomarker protein pattern analysis
HE4 (human epididymis protein 4)
Mesothelin
Oviduct-specific glycoprotein: a tissue-specific, early-stage marker for ovarian malignancies
Serum albumin-associated peptides and proteins
TIM-3 (T cell immunoglobulin and mucin protein 3)
Mutation of genes
BRCA1
P53
Gene expression studies
Tissue array analysis of gene expression: Ccne1, Ran, Cdc20 and Cks1
Gene and protein expression: CLU, ITGB3, CAPG, and PRAME
Epitomics approach for ovarian cancer biomarkers in serum
Assessment of multiple ovarian cancer biomarkers
OvPlex™ (HealthLinx): multimarker panel of ovarian cancer biomarkers:
3D microfluidic platform to assess multiple ovarian cancer biomarkers

3D Microfluidic Platform to Assess Multiple Ovarian Cancer Biomarkers

Although ovarian carcinomas are primarily metastasize within the abdominal cavity and spread to adjacent organs by direct extension prior to dissemination to distant sites, natural fluidic streams of malignant ascites triggered by gravity and negative subdiaphragmatic pressure also play a role. The role of fluidic forces as modulators of metastatic cancer biology has been investigated in a customizable microfluidic platform using 3D ovarian cancer nodules (Rizvi et al. 2013). Changes in the morphological, genetic, and protein profiles of biomarkers associated with aggressive disease were evaluated in the 3D cultures grown under controlled and continuous laminar flow. A modulation of biomarker expression and tumor morphology due to hydrodynamic forces, consistent with increased epithelial-mesenchymal transition, was observed. This is a critical step in metastatic progression and is an indicator of aggressive disease. The increase in epithelial-mesenchymal transition is driven in part by a posttranslational up-regulation of EGFR expression and activation, which is associated with the worst prognosis in ovarian cancer. A flow-induced, transcriptionally regulated decrease in E-cadherin protein expression and a simultaneous increase in vimentin was observed, indicating increased metastatic potential. These findings demonstrate that fluidic streams induce a motile and aggressive tumor phenotype. The microfluidic platform developed by these authors provides a flow-based framework that is complementary to conventional mechanism-based therapeutic strategies.

CA125 as Biomarker of Ovarian Cancer

CA125, the most widely used ovarian cancer biomarker, was first identified ~35 years ago in an antibody screen against ovarian cancer antigen. Later it was characterized to be a transmembrane mucin, MUC16, which is involved in several types of cancer. MAbs against the tandem repeat domains of MUC16, eg, oregovomab and abagovomab, have been used for targeted therapy, but had very limited success. Since the identification of CA125 to be MUC16, it is hypothesized to undergo proteolytic cleavage, a process that is considered to be critical in determining the kinetics of MUC16 shedding as well as generation of a cell-associated carboxyl-terminal fragment with potential oncogenic functions. MUC16-targeted therapy should be based understanding of the basic biologic processes involving MUC16 (Das and Batra 2015).

Epitomics Approach for Ovarian Cancer Biomarkers in Serum

A noninvasive screening test would significantly facilitate early detection of epithelial ovarian cancer. A combination of high-throughput selection and array-based serologic detection of many antigens can indicate the presence of cancer, thereby using the immune system as a biosensor. This high-throughput approach would

involve biopanning of an ovarian cancer phage display library using serum immunoglobulins from an ovarian cancer patient as bait. Protein macroarrays containing selected antigen clones reveal several that interact with immunoglobulins in sera from ovarian cancer patients but not with sera from healthy women or patients having other benign or malignant gynecologic diseases. Sequence analysis data of these clones reveals different antigens. Among the biomarkers, some known antigens have been identified, including RCAS1, signal recognition protein-19, AHNAK-related sequence, nuclear autoantogenic sperm protein, Nijmegen breakage syndrome 1 (Nibrin), ribosomal protein L4, Homo sapiens KIAA0419 gene product, eukaryotic initiation factor 5A, and casein kinase II, as well as many previously uncharacterized antigenic gene products. This global approach to antigenic profiling, epitomics, has applications in cancer and autoimmune diseases for diagnostic and therapeutic studies. Further work with larger panels of antigens should provide a comprehensive set of biomarkers with sufficient sensitivity and specificity suitable for clinical testing in high-risk populations.

xMAP technology (Luminex) is able to analyze multiple proteins in a single drop of blood or serum from women with ovarian cancer. Testing of 450 serum samples for 46 biomarkers that had previously been correlated with ovarian cancer and were able to identify a multimarker panel, comprised of 20 proteins that correctly recognized more than 98% of serum samples from women with ovarian cancer, offering higher diagnostic power than any other published assay for ovarian cancer. Their goal is to develop this screening assay into a diagnostic test to improve the early detection of ovarian cancer and to monitor therapeutic response and recurrence in women with the disease.

FGF18 as a Biomarker in Ovarian Cancer

Amplification of chromosomal region 5q31–5q35.3 has been used to predict poor prognosis in patients with advanced stage, high-grade serous ovarian cancer. A study, has further dissected this large amplicon and identified the overexpression of FGF18 as an independent predictive biomarker for poor clinical outcome in patient with ovarian cancer (Wei et al. 2013). Using cell culture and xenograft models, the authors show that FGF18 signaling promote tumor progression by modulating the ovarian tumor aggressiveness and microenvironment. FGF18 controls migration, invasion, and tumorigenicity of ovarian cancer cells through NF-κB activation, which increases the production of oncogenic cytokines and chemokines. This results in a tumor microenvironment characterized by enhanced angiogenesis and augmented tumor-associated macrophage infiltration and M2 polarization. Tumors from ovarian cancer patients have increased FGF18 expression levels with microvessel density and M2 macrophage infiltration, confirming the in vitro results of the study. In addition to gene amplification, the authors' IHC study demonstrated FGF18 overexpression in ~50% of high-grade serous tumor cases compared with benign cysts These findings demonstrate that FGF18 is important for a subset of ovarian cancers and may serve as a therapeutic target.

Gene Expression Studies in Ovarian Cancer

IHC has been used to monitor a subset of differently expressed candidate genes (Ahr, Paep, Madh3, Ran, Met, Mek1, Ccne1, Ccd20, Cks1 and Cas) in tissue arrays composed of serous ovarian tumors of different grades (0–3) and stages (I-IV). All biomarkers assayed show differential protein expression between serous tumors of low and high grade. Significant differences in Ccne1 and Ran expression are observed in a comparison of low malignant potential and grade 1 tumor samples. In addition, irrespective of the grade, Ccne1, Ran, Cdc20 and Cks1 show significant differences of expression in association with the clinical stage of disease. High levels of Ran and Cdc20 appear to be more tightly associated with a poor prognosis. The application of these biomarkers in both the initial diagnosis and prognostic attributes of patients with epithelial ovarian tumors should prove to be useful in patient management.

One study on patients with serous ovarian adenocarcinomas found that the gene and protein ITGβ3 (Integrin beta 3) were significantly more expressed in tumors from survivors compared to tumors from deceased patients, but no significant differences were detected for the other three genes or proteins CLU, CAPG, and PRAME (Partheen et al. 2009). Therefore, loss of ITGβ3 expression in tumors from deceased patients and high expression in tumors from survivors could be used as a biomarker for patients with advanced serous tumors.

HE4 Protein in Urine as a Biomarker for Ovarian Cancer

Human epididymis protein 4 (HE4) has been described as a new biomarker for the early diagnosis of ovarian cancer. HE4 protein is overexpressed in ovarian carcinomas and can be detected in serum by an ELISA with sensitivity similar to CA125 (MUC16) and higher specificity for malignant disease. HE4 is better than CA125 as a biomarker for the diagnosis of ovarian cancer and could be an important early indicator of the recurrence of the disease (Anastasi et al. 2010). HE4 can also be detected in the urine at a specificity level of 94.4%, including 86.6% of tumors at stage I/II, 89.0% at stage III/IV, and including 90.5% of patients with serous ovarian carcinoma (Hellstrom et al. 2010). Assaying urine for HE4 or mesothelin may detect early ovarian carcinoma more often than assaying serum. Antibodies to mesothelin and HE4 are more frequent in women with ovarian carcinoma or with certain types of infertility than in controls (Hellstrom and Hellstrom 2011). Their data indicate that measuring HE4 in urine may aid diagnosis and the monitoring of response to therapy. The authors anticipate that, within the next 5 years, a greater emphasis will be given to the fact that the different subtypes of ovarian carcinoma represent different types of disease. Each different type of disease will require a different diagnostic approach and more efforts will focus on high-grade serous ovarian carcinoma for which the clinical need is the greatest.

Hematogenous Metastasis of Ovarian Cancer

Ovarian cancer has a clear predilection for metastasis to the omentum, but the underlying mechanisms involved in ovarian cancer spread were not well understood. A study used OncoCEE microfluidic device (Biocept Inc), which captures CTCs and then evaluates the expression of the genomic marker HER3 in ovarian cancer cells, demonstrated preferential hematogenous metastases of ovarian cancer to the omentum (Pradeep et al. 2014). The study revealed that the ErbB3-neuregulin 1 (NRG1) axis is a dominant pathway responsible for hematogenous omental metastases. Elevated levels of ErbB3 in ovarian cancer cells and NRG1 in the omentum have enabled tumor cell localization and growth in the omentum. Depletion of ErbB3 in ovarian cancer impaired omental metastases. These results highlight hematogenous spread as an important mode of ovarian cancer metastases and use of this knowledge to design better strategies for prevention and treatment.

HtrA1 as a Biomarker of Response to Chemotherapy in Ovarian Cancer

Expression of HtrA1, which is frequently downregulated in ovarian cancer, influences tumor response to chemotherapy by modulating chemotherapy-induced cytotoxicity. Two anticancer agents, cisplatin and paclitaxel, increase the expression of the HtrA1 in ovarian carcinoma cells and thus induce cell death. Conversely, reduced HtrA1 expression reduced the effectiveness of cisplatin and paclitaxel. Patients with ovarian or gastric tumors expressing higher levels of HtrA1 show a better response to chemotherapy compared to those with lower levels of HtrA1 expression. Loss of HtrA1 in ovarian and gastric cancer may contribute to the development of resistance to chemotherapy.

Mutation of Genes in Ovarian Cancer

Sporadic ovarian tumors are the end result of a complex pathway involving multiple oncogenes and tumor suppressor genes, including HER-2/neu, K-ras, p53, BRCA1 and additional tumor suppressor genes on chromosome 17. Recent studies indicate that germline mutations of the BRCA1 gene confer a lifetime risk of approximately 45% for ovarian cancer in families with multiple cases of such cancer (this gene is also involved in breast cancer). In the general population mutations in the BRCA1 gene occur in approximately 5% of women in whom ovarian cancer is diagnosed before the age of 70 years. Protein truncation test outperforms single strand conformation polymorphism analysis for BRCA1 mutation detection in ovarian cancer.

Mutations at the p53 tumor suppressor gene locus are frequently associated with human ovarian carcinoma. A multiplex PCR screening assay has been used to amplify the complete p53 coding region from genomic DNA in a single step. Deletions and insertions were subsequently found in the newly established ovarian carcinoma cell lines.

Serum Biomarkers of Ovarian Cancer Prognosis

Serum CA125 (MUC16), the most studied biomarker for ovarian cancer, is only expressed by 50–60% of patients with early stage disease, and has a very limited value for prediction of ovarian cancer. CA-125 can be elevated by conditions other than cancer. Considerable efforts have been deployed to identify novel serum markers, yet no single biomarker has emerged as a serious competitor for CA125. The relationship between survival and early changes in the serum level of the CA-125 antigen in patients with advanced ovarian cancer is not well defined. Among women in the general US population, simultaneous screening with CA-125 and transvaginal ultrasound compared with usual care did not reduce ovarian cancer mortality (Buys et al. 2011). Diagnostic evaluation following a false-positive screening test result was associated with complications and unnecessary surgery. Pretreatment CA-125 values do not correlate with survival but the concentration of this tumor biomarker after initiation of therapy is a powerful independent prognostic factor. Reduction in the serum CA-125 concentration over the initial two cycles of platinum-based chemotherapy is a powerful independent predictor of survival for patients with suboptimal stage III or IV ovarian cancer. Patients without significant declines in CA-125 after two cycles of platinum-based chemotherapy have a particularly poor prognosis.

VEGF is a therapeutic target in ovarian cancer due to important role of angiogenesis in tumor progression. The tissue inhibitor of metalloproteinase 1 (TIMP-1) is also involved in tissue invasion and angiogenesis. High TIMP-1 and VEGF serum levels during first-line therapy of ovarian cancer patients predict poor prognosis (Mahner et al. 2010).

TIM-3 as a Biomarker of Ovarian Cancer

T cell immunoglobulin and mucin protein 3 (TIM-3) is a type I cell surface protein that was originally identified as a marker for murine T helper type 1 cells. TIM-3 was found to negatively regulate murine T cell responses and galectin-9 was described as a binding partner that mediates T cell inhibitory effects of TIM-3. A study has shown that expression of TIM-3 is significantly increased in both CD4+ and CD8+ T cells in ovarian cancer patients than in controls (Wu et al. 2013). Patients who had recurrent ovarian cancer had a higher proportion of TIM-3+CD4+ T cells than when they were newly diagnosed. Patients with a higher tumor grade demonstrated further augmented TIM-3 expression in CD4+ and CD8+ T cells compared to those with lower tumor grades. These findings suggest that TIM-3 may participate in the development and progression of ovarian cancer by its negative regulation on various T cell subsets, and TIM-3 expression in CD4+ T cells could serve as a predictive biomarker for anticancer therapies.

Multiplex Assays for Biomarkers of Ovarian Cancer

Since no single biomarker is adequate for detection of ovarian cancer, attempts have been made to use multiplex assays. Univariate and multivariate statistical analyses applied to protein-profiling data obtained from serum samples of patients with ovarian cancer using protein biochips has led to the discovery of biomarker protein panels, which can distinguish serum samples from healthy controls and patients with either benign or malignant ovarian neoplasia. Two tumor biomarkers, CA125 and HE4 (approved by FDA), are used to track whether chemotherapy is working or ovarian cancer is recurring. A one-time CA125 test can not screen seemingly healthy women because levels rise with benign cysts, endometriosis, even normal menstruation, but Fujirebio's triage test uses HE4 and CA125 to assess who most likely has a benign cyst and whose has cancer.

OvaSure (LabCorp) measures concentrations of leptin, prolactin, osteopontin, insulin-like growth factor II, macrophage inhibitory factor, and CA-125 by using a multiplex, bead-based, immunoassay system. OvaSure is a screening test for women at high risk of ovarian cancer that was developed by Yale University under a law that allows a single laboratory to offer testing without FDA review. Used on blood samples stored from cancer patients and healthy women, the test correctly identified cancer a sensitivity of 95.3% and a specificity of 99.4% (Visintin et al. 2008). However, this does not prove that OvaSure can detect when cancer is forming. Yale is working to validate OvaSure.

MS pattern analysis is a potentially rewarding approach in that it is based on power of combined multiple biomarkers so that discrimination accuracy is higher. Having reliable, discriminatory patterns obviates the need to identify and purify the biomarkers of interest and develop molecular assays for them. This process can be quite tedious, especially if the biomarkers are in low concentration. Furthermore, MS pattern assays take advantage of the high resolving power and small sample volume requirement of mass spectrometry. MS pattern analysis requires laboratories to develop new ways to continually affirm platform and sample integrity in the absence of biology-based means. Essay variability could arise from potential heterogeneity of molecules within a spot, which is why SELDI-TOF employs multiple desorptions from different positions within a spot.

Various studies have shown that a 3-biomarker panel could classify stage I/II ovarian cancer samples healthy control samples with 97% specificity and 74% sensitivity, compared to 97% specificity and 54% sensitivity when CA-125 alone was used to classify. Even though it is considerably better than use of CA-125, it does not meet the requirements for a screening test. The 3-biomarker panel would have greater value if used in conjunction with another complementary test. OvPlex™ (HealthLinx, Australia) first generation ovarian cancer 3-biomarker panel was launched in Australia with diagnostic efficiency of 92.9%. In the second generation product two new novel biomarkers were been added (HTX005 and HTX010). A phase II biomarker trial on the second generation 5-biomarker panel OvPlex™ increased the diagnostic efficiency of the panel to 98% for early stage diagnosis as compared with CA125 with diagnostic efficiency of <60% for early stage detection.

Concluding Remarks on Biomarker-Based Tests of Ovarian Cancer

The multiple ovarian cancer detection tests under development are based upon different, complementary technologies and disparate biomarkers, so in principle their combined use will provide higher accuracy. Suboptimal sensitivity of a detection assay can be compensated somewhat through regular testing of women at high risk; a convenient assay makes such routine testing less burdensome and increases patient compliance. These arguments suggest that an assay with even 95% sensitivity and specificity should help in the management of ovarian cancer.

A major advantage of isolating discrete biomarkers is that immunoassays or other biomolecule-specific assays can be developed for their detection. Immunoassays are performed routinely in the clinical lab on automated platforms with high throughput and, as such, are more economical and practical than SELDI-TOF in its present form. However, development of sufficiently sensitive immunoassays would be required.

Molecular analysis of ovarian cancer, a highly heterogeneous disease, eveals a large degree of variability among patients, and understanding this variability may be key for the development of tests able to detect various phenotypes of the disease. These differences are multifactorial and therefore investigating tumor/host interactions such as immune responses and angiogenesis may translate into the next generation of biomarkers for early detection of ovarian cancer (Seiden 2009).

Pancreatic Cancer Biomarkers

The survival rate of pancreatic cancer patients is the lowest among those with common solid tumors, and early detection is one of the most feasible means of improving outcomes. Because of the complex pathophysiology of pancreatic cancer, sensitive and specific biomarkers are required, but are lacking. “Omics” studies are being carried out to find candidate biomarkers and contribute to high-throughput systems for large cohort screening. For improvement of early diagnosis, only a panel of soluble biomarkers could provide the appropriate combination between high sensitivity and specificity (Tanase et al. 2009). A classification of biomarkers is shown in Table 13.14 and some examples of proteomic and microRNA biomarkers will be described in the following text.

Discovery and Validation of Pancreatic Cancer Biomarkers

A compendium representing biomarkers for pancreatic cancer in a global and systematic fashion was the first step (Harsha et al. 2009). It is already being used by a consortium of investigators who are developing antibodies against the 60 most promising targets. This compendium also included data on other, less common subtypes of pancreatic cancer. Because of the to mRNA-based methods, especially

Table 13.14 Classification of biomarkers of pancreatic cancer

Classical markers
Carcinoembryonic (CEA)
Carbohydrate antigen (CA19)
Mucin family (MUC)
Oncogenes: Ki-67, p53 and bcl-2
Serum biomarkers
Angiogenesis and growth factors
Circulating exosomes: glypican-1
Miscellaneous serum biomarkers: M2 pyruvate kinase, HSP27
Tissue biomarkers
Caveolin-1 (Cav-1)
Histone modifications
Fibroblast activation protein (FAP)
Maspin (Serpin B5)
Pancreatic and duodenal homeobox-1 (PDX-1)
Tissue transglutaminase 2 (TG2)
Omics biomarkers
Genomics/transcriptomics: K-ras, HIF1a, bFGF, VEGF and PDGFA
Pharmacogenetic biomarkers of response to gemcitabine: specific SNPs with prognostic value
Proteomics: UHRF1, ATP7A, aldehyde oxidase 1 (AOX1), alpha1-antichymotrypsin, human R protein
Pancreatic cancer stem cells
Signaling pathways biomarkers
MAPK and ERK pathway
TGF-β signaling pathway
Notch, Hedgehog and Wnt signaling pathways
MicroRNA biomarkers
miR-21, miR-210, miR-155, and miR-196a

DNA microarrays, 74% of the molecules in this compendium are based solely on mRNA evidence. The data requires subsequent validation by other methods. Further, several high-throughput studies carried out to identify genes that are differentially expressed in pancreatic cancer have used tissues that are not microdissected to separate cancer from stroma. Thus, it is unclear in many instances if the observed difference in the expression of a particular gene originates in the stroma or in cancer cells. This further underscores the importance of validating these observations using alternative methods by targeted studies.

Cancer Stem Cells as Biomarkers of Pancreatic Cancer

There is increasing evidence that pancreatic cancer is hierarchically organized and sustained by a distinct subpopulation of cancer stem cells (CSCs). CSCs constitute a distinct subpopulation in the tumor and are considered to drive both tumorigenesis

and metastasis; these cells are thought to be highly resistant to standard treatment modalities. Pancreatic CSCs are considered to be prognostic or predictive biomarkers as well as potential targets for therapy of pancreatic cancer (Sergeant et al. 2009). Direct evidence for the CSC hypothesis has emerged from mouse models suggesting that specific targeting of these cells is possible and therapeutically relevant (Hermann et al. 2009).

Circulating Exosomes as Biomarkers of Pancreatic Cancer

A cell surface proteoglycan, glypican-1 (GPC1), specifically enriched on cancer-cell-derived exosomes has been identified with MS analysis (Melo et al. 2015). GPC1+ circulating exosomes (crExos) were monitored and isolated using flow cytometry from the serum of patients and mice with cancer. GPC1+ crExos were detected in the serum of patients with pancreatic cancer with absolute specificity and sensitivity, distinguishing healthy subjects and patients with a benign pancreatic disease from patients with early- and late-stage pancreatic cancer. Levels of GPC1+ crExos correlate with tumor burden and the survival of pre- and post-surgical patients. GPC1+ crExos from patients and from mice with spontaneous pancreatic tumors carry specific KRAS mutations, and reliably detect pancreatic intraepithelial lesions in mice despite negative signals by MRI. GPC1+ crExos may serve as a potential non-invasive diagnostic and screening tool to detect early stages of pancreatic cancer to enable possible curative surgical therapy.

Histone Modifications Used as Biomarkers in Pancreatic Cancer

Measuring levels of specific histone modifications within cells has previously shown that low cellular levels of particular histones could determine which prostate cancer patients were more likely to suffer a recurrence and which patients with lung and kidney cancers would experience poorer survival rates. An assay to detect histone modifications can now be used to predict prognosis and response to treatment in subsets of patients with pancreatic cancer (Manuyakorn et al. 2010). The scientists used tissues from a cohort of patients enrolled in the radiation therapy oncology group (RTOG) 9704 trial, a multicenter, phase III study of pancreatic cancer comparing adjuvant gemcitabine with 5-FU, and a separate cohort of patients with stage 1 or 2 pancreatic cancer. Immunohistochemistry was performed for histone H3 lysine 4 dimethylation (H3K4me2), histone H3 lysine 9 dimethylation (H3K9me2), and histone H3 lysine 18 acetylation (H3K18ac). Positive tumor cell staining for each histone modification was used to classify patients into low- and high-staining groups, which were related to clinicopathologic parameters and clinical outcome measures. Low cellular levels of H3K4me2, H3K9me2, or H3K18ac were each significant and independent predictors of poor survival. Combined low levels of H3K4me2 and/or H3K18ac were the most significant predictor of overall survival. In subgroup analyses, histone levels were predictive of survival specifically for those patients with node-negative cancer or for those

patients receiving adjuvant 5-FU but not gemcitabine in RTOG 9704. The investigators concluded that cellular levels of histone modifications define previously unrecognized subsets of patients with pancreatic adenocarcinoma with distinct epigenetic phenotypes and clinical outcomes and represent prognostic and predictive biomarkers that could form basis of clinical decisions, including the use of 5-FU chemotherapy. Further research in cell lines and animal models will determine what, if any, role the histone modifications have in causing the development of aggressive forms of pancreatic cancer. Uncovering the mechanism of how the histone modifications are associated with cancer development and/or progression may facilitate design of strategies to interfere with that process and form the basis for a targeted therapy or chemoprevention.

miRNA Biomarkers of Pancreatic Cancer

A miRNA expression signature that is associated with pancreatic cancer has been identified (Lee et al. 2007a). This has been accomplished with the application of real-time PCR profiling of over 200 miRNA precursors on specimens of human pancreatic adenocarcinoma, paired benign tissue, normal pancreas, chronic pancreatitis and pancreatic cancer cell lines. Hierarchical clustering was able to distinguish tumor from normal pancreas, pancreatitis and cell lines. An algorithm correctly classified all tumors. One hundred miRNA precursors were aberrantly expressed in pancreatic cancer or desmoplasia, including miRNAs previously reported as differentially expressed in other human cancers (miR-155, miR-21, miR-221 and miR-222) as well as those not previously reported in cancer (miR-376a and miR-301). Most of the top aberrantly expressed miRNAs displayed increased expression in the tumor. Reverse transcription in situ PCR showed that three of the top differentially expressed miRNAs (miR-221, -376a and -301) were localized to tumor cells and not to stroma or normal acini or ducts. Aberrant miRNA expression may offer new clues to pancreatic tumorigenesis and may provide diagnostic biomarkers for pancreatic adenocarcinoma.

Microarray and qRT-PCR platforms have been used to identify miR-196a and miR-217 as the top biomarker candidates that distinguish pancreatic ductal adenocarcinoma (PDAC) from chronic pancreatitis. The qRT-PCR assay developed using this miRNA signature was validated using formalin-fixed, paraffin embedded (FFPE) pancreatic blocks and achieved 95.24% sensitivity and 94.87% specificity. Early feasibility experiments showed that the assay can also be successfully used to identify PDAC in low tissue yielding clinical specimens, such as fine needle aspirate biopsies. In addition, interrogation of microdissected populations of normal, premalignant and malignant cells revealed that miR-196a is specific to PDAC cells and can be detected as early as in PanIn-2 precursor lesions. Ongoing efforts will assess whether elevated expression of miR-196a in pancreatic tissue may enable earlier identification of patients at high risk of developing PDAC.

A modified protocol has been used to isolate and quantify plasma miRNAs from heparin-treated blood to show that miRNA in plasma can differentiate pancreatic

adenocarcinoma patients from healthy controls (Wang et al. 2009). This was used to profile 4 miRNAs – miR-21, miR-210, miR-155, and miR-196a – all implicated in the development of pancreatic cancer with either proven or predicted target genes involved in critical cancer-associated cellular pathways. Of these, miR-155 has been identified as a candidate biomarker of early pancreatic neoplasia, whereas elevated expression of miR196a has been shown to parallel progression of disease. The results revealed a sensitivity of 64% and a specificity of 89% with the analyses of plasma levels for this panel of four miRNAs. The miRNA panel could detect more than 60% of the pancreatic cancer cases. These observations, although a "proof of principle" finding at this time, show the feasibility of developing plasma miRNA profiling as a sensitive and specific blood-based biomarker assay for pancreatic cancer that has the potential of translation to the clinic with additional improvements in the future. This biomarker search is being expanded by taking a genome-wide approach to finding characteristic miRNAs in pancreatic cancer using miRNA microarrays.

Macrophage Inhibitory Cytokine-1 as Biomarker of Pancreatic Cancer

Several studies have demonstrated that the macrophage inhibitory cytokine-1 (MIC-1) may be a powerful diagnostic biomarker in patients with pancreatic cancer, but some studies are inconclusive. A metaanalysis pooled data from selected studies to yield sensitivity, specificity, positive and negative likelihood ratio (LR), diagnostic odds ratio (DOR), and receiver operating characteristic (SROC) curve (Chen et al. 2014). The results revealed that serum MIC-1 levels in pancreatic patients were higher than those of healthy subjects. The area under the SROC curve was 0.92; the pooled sensitivity was 0.79; and the pooled specificity was 0.86. The pooled positive LR was 6.20; and the pooled DOR was 35.73. Thus serum MIC-1 may be a useful diagnostic biomarker with high sensitivity and specificity for identifying pancreatic cancer.

Proteomic Biomarkers of Pancreatic Cancer

Expression Difference Mapping experiments with serum samples from patients with pancreatic adenocarcinoma compared to those with non-malignant pancreatic diseases and healthy controls have shown that the discriminative power of the resulting biomarker panels was superior to the established CA19-9 marker and the best results were achieved by using combined panels of SELDI markers together with CA19-9.

In conventional practice, the use of CA 19-9 levels and imaging techniques is not optimal for detecting small pancreatic lesions. New experimental approaches, such as quantitative proteomics, have shown great potential for the study of cancer and have opened new opportunities to investigate crucial events underlying pancreatic tumorigenesis and to exploit this knowledge for early detection and better

intervention. Isotope-coded affinity tag technology for proteomic analysis of human cancer tissue has been used to identify differentially expressed proteins in pancreatic cancer.

A specific cell receptor, the RON receptor tyrosine kinase, is a member of the MET proto-oncogene family and is important for cell proliferation, differentiation, and cancer development. RON receptor is overexpressed in pancreatic cancer cells suggesting that the receptor may also contribute to the disease's development (Thomas et al. 2007). RON receptor was active in 93% of pancreatic intraepithelial neoplasia, an early form of pancreatic duct cancer. In addition, the receptor was present in 79% of primary pancreatic cancers and 83% of metastatic cancers. RON receptor's signaling pathways could be a key factor contributing to pancreatic cancer progression. If the receptor could be blocked, it would provide a new target for RON-directed therapies that are more effective in treating this cancer.

One study has searched for pancreatic cancer biomarkers by use of high-resolution MS and acrylamide isotopic labeling in the plasma proteome of mice genetically engineered to develop cancers that closely resemble human pancreatic tumors (Faca et al. 2008). Finally, using blood samples collected during a clinical trial, the Carotene and Retinol Efficacy Trial (a cancer-prevention study), the researchers showed that the measurement of five of the proteins present in increased amounts at an early stage of tumor development in the mouse model discriminated between people with pancreatic cancer and matched controls up to 13 months before cancer diagnosis. Such proteins could be used in screening blood tests for early and more accurate detection of cancer.

Concluding Remarks on Biomarkers of Pancreatic Cancer

There are several diagnostic methods available to pancreas including CT scan, gadolinium-enhanced MRI, magnetic resonance cholangiopancreatography and endoscopic ultrasound. However, the most promising method is endoscopic ultrasound-guided fine-needle aspiration as this technique enables analysis of cyst fluid using biomarkers (Moris et al. 2017). Previously two biomarkers were available in clinical practice: carcinoembryonic antigen and carbohydrate antigen 19-9. Now DNA analysis of pancreatic cystic fluid and genomic analysis may provide new tools for the diagnosis. Novel genomic and serum biomarkers could play an important future role to identify those individuals who will benefit from an early operation and those who will benefit from watchful waiting approach.

Parathyroid Cancer Biomarkers

Parathyroid carcinoma (PC) is a rare malignancy and causes primary hyperparathyroidism (HPT) with high morbidity and mortality in advanced cases usually resulting from intractable hypercalcemia. Inactivation of the HRPT2/CDC73 gene,

encoding the putative tumor-suppressor protein parafibromin and discovered in the context of the hyperparathyroidism-jaw tumor (HPT-JT) syndrome, is a common, somatic event in most parathyroid cancers. Approximately 25% of patients with apparently sporadic parathyroid cancer carry germline HRPT2/CDC73 mutation (Sharretts et al. 2010). Therefore, germline DNA analysis for HRPT2/CDC73 mutation is recommended in all patients with parathyroid cancer because of the potential benefit for first-degree relatives, who should nevertheless undergo serum calcium screening.

PC is usually not recognized preoperatively and is often not conclusively identified during surgery. There is a need for biomarkers of PC. Currently, several issues still need to be addressed, such as the lack of common criteria for the histopathological diagnosis of PC and preoperative diagnosis. The latter is important for the surgeon in deciding for a complete resection of all cancerous tissue at the time of the initial surgery with the increased likelihood of a cure (Falchetti et al. 2012).

Peripheral Nerve Tumors

Biomarkers of Neurofibromatosis

Neurofibromatosis type 1 (NF1) is a hereditary tumor syndrome characterized by the development of benign nerve-sheath tumors, which transform to malignant peripheral nerve-sheath tumors (MPNST) in about 8 to 13% of patients with NF1. NF1 is the most common type of neurofibromatosis NF1 is the most common type of neurofibromatosis MPNST are invasive sarcomas with extremely poor prognosis, and their development may correlate with internal tumor load of patients with NF1. Because early identification of patients with NF1 at risk for developing MPNST should improve their clinical outcome.

A study has aimed to identify serum biomarkers for tumor progression in NF1, and to analyze their correlation with tumor type and internal tumor load (Park et al. 2013). Analysis of results identified 4 biomarkers (EGFR, IFN-γ, IL-6, and TNF-α) for which significantly different serum concentrations were seen in patients with NF1 compared with healthy controls. Two biomarkers, IGFBP1 and regulated upon activation, normal T-cell expressed and secreted (RANTES), showed significantly higher concentrations in patients with NF1 and MPNST compared with patients with NF1 without MPNST. A correlation with internal tumor load was found for IGFBP1. Moreover, the data suggest that there is a systemic increase in inflammatory cytokines independently of tumor load in patients with NF1.

Although research has provided some information about the molecular mechanisms of tumor development in NF1, few if any biomarkers exist for the prognosis of the disease. In 2015, the National Biomarker Development Alliance and the Children's Tumor Foundation in USA formed a partnership aimed at advancing biomarkers for neurofibromatosis. The partners will create a center tasked with discovering and developing biomarkers of pediatric brain tumors with a focus on NF1-

associated tumors. The center will use an evidence-based approach to evaluate biomarkers, prioritize them for development, and move them into qualification and validation studies. Qualification studies will be pursued under the FDA biomarker qualification program. The center will make its data and processes publicly available to the broader research community for work into all pediatric brain tumors.

Prostate Cancer

Prostate cancer is the most common cause of death from cancer in men over the age of 75. Over 230,000 new prostate cancer cases and 30,000 prostate cancer deaths are estimated in the US per year. This makes prostate cancer the most commonly diagnosed cancer and the second leading cause of cancer-related deaths in men in the US. Various biomarkers of cancer of the prostate are listed in Table 13.15.

Table 13.15 Biomarkers of prostate cancer

Antigens
Prostate specific antigen
Prostate-specific membrane antigen
Adipose tissue-derived biomarkers of obesity-related prostate cancer
Adipokines
Metabolomic biomarkers of prostate cancer
Sarcosine
Prostate biomarkers in body fluids
Biomarker in semen
Biomarker in urine: Annexin 3; EPCA-2, sarcosine
Exosomes as biomarkers in serum and urine
mRNA biomarkers in serum and urine
HAAH in serum
Prostasomes in serum
Serum-protein fingerprinting
Biomarkers of prostate cancer in biopsy specimens
Expression of proteins: hepsin and PIM1
Genes relevant to diagnosis of prostate cancer
Detection of DD3 (PCA3) gene in urine
Genetic biomarkers of prostate cancer
Kallikrein gene polymorphisms: KLK2, KLK3, etc
Tests for prostate cancer based on genetic dislocations
Tests for prostate cancer based on gene expression
Epigenetic biomarkers of prostate cancer
miRNA biomarkers of prostate cancer
Prostate Health Index

Adipose Tissue-Derived Biomarkers of Obesity-Related Prostate Cancer

Obesity (adiposity) is associated with prostate cancer, particularly with its accelerated progression. Adipose factors including adiponectin, leptin, IGF, TNF-α, and IL-6, are molecular mediators between prostate cancer and obesity. They stimulate prostate cancer cell growth and subsequent blockage of these adipose factors will suppress androgen-independent prostate cancer cell growth and increase survival. These adipose factor functions can be blocked by inhibiting their expression, the expression of their cell surface receptors, or by inhibiting the binding of these cytokines to their receptors. Adipokines may contribute to the molecular basis for the association between obesity and prostate cancer, but the complex pathophysiology of both these disease states requires further studies.

B7-H3 as Biomarker of Prostate Cancer

Until now there were no strongly-predictive molecules for prostate cancer. PSA and PSMA are useful for diagnosis of prostate cancer. However, PSA tends to leave prostate cancer cells and migrate throughout the body, making it a poor target for therapy. Mayo Clinic researchers have identified B7-H3 as the first immune molecule that appears to play a role in prostate cancer development and in predicting cancer recurrence and progression after surgery (Roth et al. 2007). Expression levels of B7-H3 in prostate cancer specimens of men treated for clinically localized prostate cancer were correlated with pathologic indicators of aggressive cancer as well as clinical outcome and B7-H3 was uniformly and aberrantly expressed by adenocarcinomas of the prostate. B7-H3 is expressed by benign prostatic epithelia, although at a more reduced level relative to neoplastic tissue. Because B7-H3 kills or paralyzes immune cells that are trying to attack the cancer, it encompasses a novel diagnostic, prognostic and even a therapeutic target for the clinical management of prostate cancer. B7-H3 represents an independent predictor of prostate cancer progression following surgery. This discovery will allow physicians to individualize treatment and observation plans for prostate cancer patients. Being able to tell a patient his specific risk after surgery, and perhaps even prior to surgery, will be a huge step forward. Evaluation of B7-H3 levels in prostate biopsies from patients may help to determine which patients may benefit from a watchful waiting strategy versus early aggressive treatment. Using this molecular signature will facilitate, for the first time, a truly individualized approach to prescribing the most appropriate therapy for a given patient, i.e. personalized medicine.

Cancer Genetics-Guided Biomarker Signatures of Prostate Cancer

A two-stage strategy has been described for the discovery of serum biomarker signatures corresponding to specific cancer-causing mutations and its application to prostate cancer in the context of the commonly occurring PTEN tumor-suppressor

gene inactivation (Cima et al. 2011). In the first stage of this approach, 775 N-linked glycoproteins were identified from sera and prostate tissue of wild-type and Pten-null mice. Using label-free quantitative proteomics, it was shown that Pten inactivation leads to measurable perturbations in the murine prostate and serum glycoproteome. In the second stage, following bioinformatic prioritization, targeted proteomics was applied to detect and quantify 39 human ortholog candidate biomarkers in the sera of prostate cancer patients and control individuals. The resulting proteomic profiles were analyzed by machine learning to build predictive regression models for tissue PTEN status and diagnosis and grading of prostate cancer. This approach suggests a general path to rational cancer biomarker discovery and initial validation guided by cancer genetics and based on the integration of experimental mouse models, proteomics-based technologies, and computational modeling.

Detection of Prostate Cancer Biomarkers in Urine

Urine is considered to be an attractive body fluid in which to pursue the identification of novel biomarkers of prostate cancer. Some proteomic techniques including MS and the newer, quantitative proteomic strategies have been applied to novel urinary biomarker identification in prostate malignancy.

PROGENSA™ PCA3 (Hologic Inc), a urine test for prostate cancer based on detection of differential display code 3 or DD3(PCA3) gene, is the first gene-based, adjunctive screen for this prostate cancer. PCA3 is over-expressed only in malignant prostate tissue and not in any other normal human tissue, including breast, bladder, testis, gastrointestinal organ, and musculoskeletal tissue. A quantitative RT-PCR assay for PCA3, which shows many-fold up-regulation of PCA3 in patients with prostate cancer, assay would be a valuable tool for the detection of malignant cells in blood, urine, or other clinical specimens, and it could have important implications for the earlier diagnosis and molecular staging of prostate cancer. The test offers advantages over PSA testing, the current standard for initial prostate cancer screening in conjunction with a digital rectal exam. It has great potential in reducing the number of unnecessary prostate biopsies.

PCA3Plus (Bostwick Laboratories) is a urine-based test for prostate cancer risk, which detects PCA3 in cells that are shed in urine. The assay measures the expression of mRNA from the PCA3 gene. The higher the PCA3Plus value, the higher a patient's risk for prostate cancer.

A multiplex panel of urine transcripts was shown to outperform PCA3 transcript alone for the detection of prostate cancer (Laxman et al. 2008). The test is based on the finding that gene fusions – pieces of chromosomes that trade places with each other, causing two genes to stick together – are common in prostate cancer, and that by overriding molecular switches that turn off excess growth, they may be the causative factor in some forms of the disease. The investigators considered six gene fusions in addition to PCA3 as putative prostate cancer biomarkers: TMPRSS2, which fuses with either ERG or ETV1, two genes known to be involved in several types of cancer and another five genes that fuse on to ERG or ETV1 to cause prostate cancer. The expression the of these seven biomarkers was measured in

sedimented urine using quantitative PCR from patients presenting for biopsy or radical prostatectomy. Univariate analysis showed that increased GOLPH2, SPINK1, and PCA3 transcript expression and TMPRSS2:ERG fusion status were significant predictors of prostate cancer. Multivariate regression analysis showed that a multiplexed model, including these biomarkers, outperformed serum PSA or PCA3 alone in detecting prostate cancer. The urine test that screens for the presence of four different RNA molecules accurately identified over 75% of patients (5% better than with PCA3 alone) who were later found to have prostate cancer, and was 61% effective in ruling out disease in other study participants. These results provide the basis for the development of highly optimized, multiplex urine biomarker tests for more accurate detection of prostate cancer.

A study has shown that incorporation of two prostate cancer-specific biomarkers – TMPRSS2:ERG and PCA3 – measured in the urine improves on serum PSA (or a multivariate risk calculator) for predicting the presence of prostate cancer and high-grade on biopsy (Tomlins et al. 2016). A combined test, Mi-Prostate Score (MLabs), uses models validated in this study and is clinically available to provide individualized risk estimates.

Studies have shown that Annexin 3 (ANXA3) quantification in urine is a novel, noninvasive test with high specificity for prostate cancer. bioMérieux and ProteoSys are collaborating to develop a confirmatory urine test for prostate cancer based on Annexin 3, which will reduce the number of unnecessary biopsies (Köllermann et al. 2008).

Detection of Prostatic Intraepithelial Neoplasia

High grade prostatic intraepithelial neoplasia (PIN) has been established as a premalignant lesion of the prostate that has a high potential to progress to invasive prostate cancer. Approximately 1.4 million prostate biopsies are performed every year in the US to detect new cases of prostate cancer. High grade PIN is found in an average of 9% of prostate biopsies which represents an estimated 125,000 new cases of high grade PIN diagnosed each year. Patients who are found to have high grade PIN are at high risk of prostate cancer with up to 37% of patients being later diagnosed with prostate cancer within one year.

PIN may prove to be an important diagnostic indicator of cancer of prostate. The development of assays for the accurate detection of PIN could be a component in the treatment of this disease. A proprietary panel of proPSA serum biomarkers from Beckman Coulter may help diagnose the presence of PIN and the earliest progression to prostate cancer.

Epigenetic Biomarkers of Prostate Cancer

In contrast to gene tic changes that may vary, certain epigenetic changes are highly consistent, in particular hypermethylation of a specific set of genes, and others regularly associated with progression, such as global DNA hypomethylation, certain

chromatin modifications and altered levels and composition of polycomb complexes. Although changes in polycombs and DNA methylation both accompany the progression of prostate cancer, they do not cause one another. However, they may contribute to establishing and maintaining an aberrant differentiation potential of prostate cancer initiating cells. Global DNA hypomethylation in prostate cancer may relate to adaptative changes in several signaling pathways typical of this cancer type, including innate immunity responses. Adaptative changes in the expression and function of chromatin regulators required to diminish the dependency of prostate cancer cells on androgens may shape the epigenome, beyond individual genes regulated by the androgen receptor (Schulz and Hoffmann 2009). Because of their crucial role, epigenetic biomarkers may become highly useful for diagnostics and therapy of prostate cancer.

Hypermethylated DNA can be detected in body fluids from prostate cancer patients and may be a useful biomarker, although clinical performance varies between studies. Real-time PCR was used to measure four DNA methylation biomarkers: GSTP1 and three previously unreported candidates associated with the genes RASSF2, HIST1H4K, and TFAP2E in sodium bisulfite-modified DNA (Payne et al. 2009). Analysis of all biomarkers in urine DNA significantly discriminated prostate cancer from biopsy negative patients. The biomarkers provided information independent of PSA and may warrant inclusion in nomograms for predicting prostate biopsy outcome. The biomarkers' prostate cancer sensitivity was greater for urine than plasma DNA. The biomarker performances in urine DNA should next be validated in formal training and test studies.

Exosomes as Biomarkers of Prostate Cancer

Discovery of novel biomarkers for prostate cancer (PCa) in complex fluids, such as serum and urine, remains a challenge. Several cancer-derived proteins and RNAs are secreted through small vesicles known as exosomes, which are potential biomarkers for PCa (Duijvesz et al. 2011). Purification of prostate- and PCa-derived exosomes will enable us to profile exosomes, providing a promising source of protein and RNA biomarkers for PCa. This profiling will contribute to the discovery of novel biomarkers for the early diagnosis and reliable prognosis of PCa.

A novel affinity-based proteomics technology examines the protein signature of exosomes by using protein binding reagents called SOMAmers® (slow off-rate modified aptamers) from SomaLogic and allows the simultaneous precise measurement of >1000 proteins (Webber et al. 2014). Exosomes are highly purified from the Du145 PCa cell line, by pooling selected fractions from a continuous sucrose gradient (within the density range of 1.1 to 1.2 g/ml), and examined under standard conditions or with additional detergent treatment by the SOMAscan™ array (version 3.0). Lysates of Du145 cells are also prepared, and the profiles are compared. Housekeeping proteins such as cyclophilin-A, LDH, and Hsp70 are present in exosomes, and ~100 proteins have been identified that are enriched in exosomes relative to cells. These include proteins of known association with cancer exosomes such as MFG-E8, inte-

grins, and MET, and also those less widely reported as exosomally associated, such as ROR1 and ITIH4. Several proteins with no previously known exosomal association are confirmed as exosomally expressed in experiments using individual SOMAmer® reagents or antibodies in micro-plate assays. Western blotting has confirmed the SOMAscan™-identified enrichment of exosomal NOTCH-3, L1CAM, RAC1, and ADAM9. Over 300 proteins of hitherto unknown association with PCa exosomes were found indicating that the SOMAmer®-based assay is an effective proteomics platform for exosome-associated PCa biomarker discovery.

Gene Expression Analysis of Prostate Cancer

Gene expression levels have compared with two reference samples – normal prostate tissue from men with prostate cancer and prostate samples from men with no history of prostate disease. Expression of two proteins, hepsin (the membrane-spanning serine protease) and PIM1 (an oncogenic kinase), is significantly correlated with poor prognosis in prostate cancer. These biomarkers are unlikely to replace PSA in the clinic for routine screening, as taking a blood sample is far less invasive than a biopsy; however, when biopsy material is available, these cell-associated makers might be useful for identifying candidates for prostatectomy.

One novel prostate cancer gene identified by gene expression analysis is hepcin – a serine transmembrane protease that is overexpressed in cancer cells from both primary and metastatic tumors. Another gene, AMACR, is upregulated only in localized prostate cancer tumors, but not metastatic. This gene is more reliable than PSA for identifying aggressive prostate tumors; when gene expression patterns of additional prostate cancer patients were evaluated, AMACR expression alone can predict, which patients had aggressive prostate tumors, with 97% specificity and 100% sensitivity.

Quantitative RT-PCR assays show that a four-gene expression signature for prostate cancer cells (consisting of UAP1, PDLIM5, IMPDH2, and HSPD1) can detect Gleason grade 3 and grade 4 cancer cells in prostate tissue (Guyon et al. 2009). This may be useful as an adjunct test to the pathology examination of prostate tissue taken at biopsy or prostatectomy.

Significant and widespread differences in gene expression patterns exist between benign and malignant growth of the prostate gland. Gene expression analysis of prostate tissues should help to disclose the molecular mechanisms underlying prostate malignant growth and identify molecular biomarkers for diagnostic, prognostic, and therapeutic use.

Genetic Biomarkers of Prostate Cancer

A study has to analyzed 12 SNPs in genes ELAC2, RNASEL and MSR1 as biomarkers for detection and progression of prostate cancer, as well as perform a genetic classification of high-risk patients (Alvarez-Cubero et al. 2015). This study

provides the proof-of-principle that some of the genetic variants (such as rs486907, rs627928 and rs2127565) in genes RNASEL, MSR1 and ELAC2 can be used as predictive biomarkers of aggressiveness and progression of prostate cancer. Clinical application of these biomarkers, in combination with current ones, could reduce the rate of unnecessary biopsies and specific treatments.

ProstaVysion (Bostwick Laboratories) is a prognostic tissue-based genetic panel, which examines two major mechanisms of prostate carcinogenesis: ERG gene fusion/translocation and the loss of the PTEN tumor suppressor gene. ERG gene fusions, found in 40% of primary prostate cancers, are associated with a more aggressive phenotype (Carver et al. 2009). They correlate with cancer stage, Gleason score, grade and cancer-specific survival rates. PTEN, is a key tumor suppressor gene in prostate cancer. Deletion of PTEN occurs in 20–40% of localized prostate cancers and up to 60% of metastases (Han et al. 2009).

By examining these two biomarkers, ProstaVysion is able to provide a molecular analysis of prostate cancer aggressiveness and long-term patient prognosis. The ProstaVysion Score summarizes patient results with information present in published medical literature for a projected outcome of prostate cancer. These results are useful to physicians for guiding patient treatment decisions.

Identification of Prostate Cancer mRNA Biomarkers

RT-PCR differential display method has been used initially to identify mRNA transcripts differentially expressed in tumor versus patient-matched nontumor prostate tissue. Averaged differential expression (ADE) of pooled tissue samples was used to identify mRNA transcripts that are differentially expressed in most tumor specimens (Bai et al. 2007). Differentially expressed mRNA transcripts identified by ADE were fewer in number, but were expressed in a greater percentage of tumors (>75%) than those identified by differential display of mRNA from individual patient samples. Differential expression of these mRNA transcripts was also detected by RT-PCR in mRNA isolated from urine and blood samples of prostate cancer patients. These findings demonstrate the principle that specific cDNA probes of frequently differentially expressed mRNA transcripts identified by ADE can be used for the detection of prostate cancer in urine and blood samples.

Kallikreins as Biomarkers of Prostate Cancer

Polymorphisms associated with prostate cancer include those in three genes encoding major secretory products of the prostate: KLK2 (encoding kallikrein-related peptidase 2; hK2), KLK3 (encoding prostate-specific antigen; PSA), and MSMB (encoding beta-microseminoprotein). PSA (hK3) is one of the human kallikreins, and is the most used tumor biomarker for prostate cancer screening, diagnosis, prognosis and monitoring. hK2, another prostate-specific kallikrein, has also been proposed as a complementary prostate cancer biomarker.

Genotyping of SNPs in "Cancer Prostate in Sweden 1" with independent replication in "Cancer Prostate in Sweden 2" study showed that a T allele at rs198977 in KLK2 was associated with increased cancer risk and a striking decrease of hK2 levels in blood (Klein et al. 2010). Based on this strong association, the investigators developed a model for predicting prostate cancer risk from standard biomarkers, rs198977 genotype, and rs198977 x hK2 interaction; this model had greater accuracy than did biomarkers alonea.

Multiple kallikrein forms measured in blood can predict the result of biopsy in previously unscreened men with elevated PSA. A multivariable model can determine which men should be advised to undergo biopsy and which might be advised to continue screening, but defer biopsy until there was stronger evidence of malignancy. The 4Kscore test (OPKO Health Inc) relies on the measurement of four prostate-specific kallikreins in blood: total PSA, free PSA, intact PSA, and human kallikrein 2 (hK2). The blood test results are combined in an algorithm with patient age, DRE (nodules, no nodules), prior negative biopsy (yes/no). The result is a patient specific probability for finding a high-grade, Gleason score 7 or higher prostate cancer upon biopsy. Use of this test could eliminate unnecessary prostate biopsies. With advances in genomics, proteomics, and other biotechnologies, the role of KLKs in prostate cancer will be further elucidated in the future to provide novel biomarker for improving screening, diagnosis, prognosis, and survival of patients (Hong 2014).

LCM for Diagnosis of Prostate Cancer

The proportion of unbound serum prostate-specific antigen (PSA; percent-free PSA) is reported to be lower in men with prostate cancer compared to men with benign prostates. The majority of immunoreactive PSA in serum is complexed to alpha-1-antichymotrypsin (ACT). Laser capture microdissection (LCM) has been used to assess the bound versus free form of intracellular PSA in both benign and malignant epithelium procured from prostate tissue. Western blotting analysis of 1D PAGE gels revealed that in the vast majority of intracellular tumor, normal PSA exists within cells in the "free" form. Binding studies showed that PSA recovered from LCM-procured cells retained the full ability to bind ACT, and 2D PAGE Western analysis demonstrated that the PSA/ACT complex was stable under strong reducing conditions. Intracellular PSA, therefore, exists in the "free" form and that binding to ACT occurs exclusively outside of the cell.

PSA or histological examination of bulk tissue may not accurately reflect molecular events that take place in the actual ductal epithelium that change as a consequence of the malignant process in the prostate gland. Alternative proteomic-based approaches including LCM enable the identification of protein biomarkers in the actual premalignant and frankly malignant epithelium.

The phenotype of a given cell type is ultimately determined by the composition and activation status of its proteins. Therefore, quantitative and qualitative proteomic measurement of normal and neoplastic prostate cells is an important

experimental approach that will complement genomic DNA and gene expression analyses. The National Cancer Institute Prostate Group has been studying protein profiles of prostate cancer using tissue microdissection and two protein analysis methods: 2D PAGE and SELDI.

SELDI ProteinChip MS technology has been used for the rapid, reproducible and simultaneous identification of four well-characterized prostate cancer-associated biomarkers: PSA (free and complexed forms), prostate specific peptide, prostate acid phophatase and prostate specific membrane antigen in cell lysates, serum and seminal plasma. Proteins corresponding to the mass of these biomarkers could readily be captured and detected using either chemically defined or antibody coated ProteinChip arrays. Several other proteins were found upregulated in cell lysates of pure populations of prostate cancer-associated cells procured by LCM when compared with mass spectra of normal cell lysates. Coupling LCM with SELDI provides tremendous opportunities to discover and identify the signature proteins associated with each stage of tumor development.

Microarray for Diagnosis of Prostate Cancer

Microarray technology has been used to analyze the molecular differences between tissue from unaffected individuals, and those with benign prostatic hyperplasia (BPH), primary localized prostate cancer or metastatic hormone-refractory prostate cancer. Gene expression levels were compared with two reference samples — normal prostate tissue from men with prostate cancer and prostate samples from men with no history of prostate disease. Expression of two proteins, hepsin (the membrane-spanning serine protease) and PIM1 (an oncogenic kinase), is significantly correlated with poor prognosis in prostate cancer. These biomarkers are unlikely to replace PSA in the clinic for routine screening, as taking a blood sample is far less invasive than a biopsy; however, when biopsy material is available, these cell-associated makers might be useful for identifying candidates for prostatectomy.

miRNA Biomarkers of Prostate Cancer

Optimized high-throughput miRNA expression profiling offers novel biomarker identification from prostate cancer biopsies enabling distinction of advanced and metastatic prostate cancers from pooled normal prostatic samples and from a non-malignant precursor lesions. Hierarchical clustering of the prostate tumor samples according to their miRNA expression can separate the carcinomas from the benign prostate hypertrophy and also further classify the carcinomas according to their androgen dependence (Porkka et al. 2007). This indicates that miRNAs expression profiling is a potential diagnostic and prognostic tool for prostate cancer. miR-221, which regulates proliferation, apoptosis, and invasion of prostate cancer cells by inhibiting IRF2 and SOCS3, has significant potential as a prognostic biomarker and

therapeutic target for improving clinical management of patients with aggressive prostate cancer (Kneitz et al. 2014).

miRNAs originating from human prostate cancer xenografts enter the circulation, are readily measured in plasma, and can robustly distinguish xenografted mice from controls. This concept extends to cancer in humans, where serum levels of miR-141 (a miRNA expressed in prostate cancer) can distinguish patients with prostate cancer from healthy controls (Mitchell et al. 2008).

A multiplex RT-qPCR method involving the purification of PCR products followed by uniplex analysis on a microfluidics chip was developed to evaluate to identify serum miRNAs that help diagnosis and correlate with the prognosis of prostate cancer (Moltzahn et al. 2011). By profiling sera from healthy men and untreated prostate cancer patients with differing CAPRA scores, the investigators identified miRNA signatures including oncogenic and tumor-suppressive miRNAs that enabled diagnosis of prostate cancer and correlation with prognosis as well as cancer progression. Some dysregulated miRNAs are predicted to target kallikreins and the miRNA-kallikrein axis of interaction provides a new factor in the pathogenesis of prostate cancer (White et al. 2012).

Extracellular miRNAs exist in different forms - associated with Ago2 proteins, loaded into extracellular vesicles (exosomes, microvesicles, or apoptotic bodies) or into high density lipoprotein particles. Potential use of miRNAs from extracellular vesicles as biomarkers for prostate cancer is under consideration (Hessvik et al. 2013).

Prostate Cancer Biomarkers in Semen

Seminal fluid (SF) is composed of secretions from glands in the male urogenital tract: 40% of SF is prostatic material, released following global smooth muscle contraction and expulsion into the urethra, with the remainder contributed by the seminal vesicles and testes. SF has potential as a clinically relevant 'liquid biopsy' of the prostate that could assist in diagnosis of prostate cancer.

PSA was originally discovered in SF, where it exists at a concentration ~5 to 6 orders of magnitude higher than in blood serum. The proportion of free- and complex-PSA in serum is used for differentiating between benign and malignant prostate disease. To further understand the physiological relationship between PSA in seminal plasma and blood, free-PSA (fPSA) and complex-PSA (cPSA) have been analyzed in blood and in SF. fPSA in blood, but not cPSA, is associated to PSA in SF. In blood cPSA, but not fPSA, increases with age in healthy men, which may reflect an increasing incidence of prostate disease.

Proteomic profiling of SF using 2DGE and MALDI-TOF/MS has identified proteins implicated in prostate cancer (Drake and Kislinger 2014). Capillary electrophoresis mass spectrometry, used to profile SF samples from men with with a panel of 11 proteins, accurately distinguish between localized and advanced prostate cancer (Neuhaus et al. 2013). Another study showed that Dkk-3, an abundant protein in SF, is found at significantly higher levels in patients with biopsy-con-

firmed prostate cancer, and the combination of Dkk-3 with serum and SF PSA demonstrated improved diagnostic accuracy compared with serum PSA alone (Zenzmaier et al. 2011).

miRNAs are also present in SF. RNA sequencing was used to profile small RNAs in the nonsperm epithelial cell fraction of SF and identified a panel of putative miRNA biomarkers of prostate cancer. miR-200b in combination with serum PSA was shown to exhibit a significantly higher diagnostic value for prostate cancer than serum PSA alone (Selth et al. 2014). The cell-free fraction of SF, including prostate-derived extracellular vesicles or 'prostasomes', is a rich source of highly stable miRNA and other molecular biomarkers of prostate cancer that is shielded from enzymatic degradation (Roberts et al. 2015).

Metabolites in SF may also be useful biomarkers of prostate cancer. Metabolic homeostasis is perturbed, leading to reduced intracellular and seminal citrate and zinc, a phenomenon that is thought to occur prior to histological changes. Changes in other metabolites in SF, such as spermine and myo-inositol, have been demonstrated using NMR spectroscopy (Roberts et al. 2011).

PSA as Biomarker of Prostate Cancer

At present, measurement of serum prostate-specific antigen (PSA) is the most useful biomarker for early detection, clinical staging and monitoring. However, although on average men with prostate cancer have higher levels of PSA than healthy men or those with benign prostate diseases, there is a wide variation in levels throughout the population, which leads to false positives and unnecessary biopsies. Also PSA test cannot reliably distinguish between prostate conditions such as benign prostatic hyperplasia and cancer. A low PSA is not a guarantee of disease-free status, and an elevated PSA is frequently associated with a negative biopsy. Of the 25 million men tested for PSA, one million will undergo prostate biopsy based upon elevated levels of PSA. It is estimated that 75% of these biopsies are unnecessary, a significant portion of which could be avoided if a more accurate diagnostic tool is available. A case-control study has shown that men at age 60 with a PSA level below 1 ng/ml, which is about half of all men, had a 0.2% chance of death from prostate cancer, are at low risk of prostate cancer death and may not need to be screened in the future (Vickers et al. 2010). The study also indicated that some men found to be at low risk may actually have prostate cancer; however it is not likely to cause symptoms or shorten their life by the age of 85.

ProPSA as Biomarker of Prostate Cancer

ProPSA is present in prostate tumor tissue but not in normal prostate and higher levels in serum indicate development of an aggressive form of prostate cancer with an unfavorable prognosis. In a prospective cohort of men enrolled into expectant management for prostate cancer, serum and tissue levels of proPSA at diagnosis are

associated with need for subsequent treatment (Makarov et al. 2009). The increase in serum proPSA/% free PSA (fPSA) might be driven by increased proPSA production from premalignant cells in the prostate benign adjacent areas. A prospective study has demonstrated that proPSA provides improved discrimination between prostate cancer and benign disease in screened men with a PSA of 2.5–10 ng/ml and a negative digital rectal examination (Le et al. 2010).

Prostate Health Index

Prostate Health Index (PHI) involves combination of results of serum total PSA, fPSA and proPSA measured by the Beckman Coulter immunoassay. All the three are analyzed simultaneously in the same serum sample. Values of PHI in general population are 33 and if >44 are associated with 44% risk of prostate cancer. As a simple blood test, it significantly improves on PSA in the selection of men for biopsy and is a major advance in prostate cancer risk assessment. It has been approved by the FDA and in several other countries. In multiple prospective international trials, this composite measurement has been shown to outperform conventional PSA and free PSA measurements. Unlike PCA3 and TMPRSS2:ERG, PHI is also consistently associated with Gleason score and upgrading during active surveillance. PHI should be considered as part of the standard urologic armamentarium for biopsy decisions, risk stratification and treatment selection (Loeb and Catalona 2014).

Prostasomes in Blood as Biomarker of Prostate Cancer

Prostasomes are microvesicles (mean diameter, 150 nm) that are produced and secreted by normal as well as malignant prostate acinar cells. In prostate cancer, rather than ending up in semen, prostasomes are pumped out into the surrounding tissue in invasive cancer. Therefore, prostasomes levels are expected to be elevated in blood in patients with prostate cancer and correlate more closely with the severity of the disease than do PSA levels. Prostasomes can be detected by proximity ligation for effective determination of proteins. In this method, the target is first captured via an immobilized antibody and subsequently detected by using four other antibodies with attached DNA strands. The requirement for coincident binding by five antibodies to generate an amplifiable reporter results in both increased specificity and sensitivity. The assay successfully detected significantly elevated levels of prostasomes in blood samples from patients with prostate cancer before radical prostatectomy, compared with controls and men with benign biopsy results (Tavoosidana et al. 2011). This approach that enables detection of prostasomes in peripheral blood may be useful for early diagnosis and assessment of prognosis in organ-confined prostate cancer.

PSMA as Biomarker of Prostate Cancer

Prostate prostate-specific membrane antigen (PSMA) in prostate tissue is increased in patients with more aggressive features, i.e. higher Gleason grade and higher stage. Significant up-regulation of PSMA expression occurs in patients with metastatic disease as compared to those with localized prostate cancer and in localized disease compared to benign prostate tissue. High PSMA levels in primary prostate cancer not only correlate with other adverse traditional prognostic factors, but can independently predict both a higher incidence and shorter time to disease recurrence.

Increased PSMA expression in response to treatment with antiandrogen drugs such as MDV3100 and abiraterone, can be quantitatively measured in vivo in human prostate cancer xenograft models through PET imaging with a fully humanized, radiolabeled antibody to PSMA, 64Cu-J591 (Evans et al. 2011). This could serve as a biomarker of androgen receptor (AR) signaling to noninvasively evaluate AR activity in patients with castration-resistant prostate cancer.

Sarcosine as a Metabolic Biomarker of Prostate Cancer

Sarcosine, an N-methyl derivative of the amino acid glycine, has been identified as a differential metabolite that is elevated during prostate cancer progression to metastasis and can be detected noninvasively in urine (Sreekumar et al. 2009). Sarcosine levels are also increased in invasive prostate cancer cell lines relative to benign prostate epithelial cells. Knockdown of glycine-N-methyl transferase, the enzyme that generates sarcosine from glycine, attenuates prostate cancer invasion. Addition of exogenous sarcosine or knockdown of the enzyme that leads to sarcosine degradation, sarcosine dehydrogenase, induced an invasive phenotype in benign prostate epithelial cells. Androgen receptor and the ERG gene fusion product coordinately regulate components of the sarcosine pathway. Profiling of the metabolomic alterations of prostate cancer progression, has revealed sarcosine as a potentially important metabolic intermediary of cancer cell invasion.

Silenced CDH13 Gene as a Biomarker of Cancer

Biochemical (prostate-specific antigen) recurrence of prostate cancer after radical prostatectomy remains a major problem. Better biomarkers are needed to identify high-risk patients. A methylation-specific PCR assay has been used to assess the methylation state of 15 genes known to influence prostate cancer in prostate cancer tissue samples taken from patients during surgery to remove all or part of the prostate gland (Alumkal et al. 2008). Prostate cancer recurrence, which occurred in one third of patients within 5 years of their surgery, was linked to silencing of one of these genes, CDH13, which codes the protein cadherin 13 (plays a role in cell-cell adhesion). The results of this study showed that methylation of CDH13 is

independently associated with an increased risk of biochemical recurrence after radical prostatectomy even considering the weighted risk of recurrence score. These findings should be validated in an independent, larger cohort of patients with prostate cancer who have undergone radical prostatectomy. Promoter methylation of CDH13, which occurs frequently in the serum of patients with prostate cancer, is associated with an increased risk of death, and may become a useful independent predictor of a poor prognosis (Wang et al. 2014).

Serum-Protein Fingerprinting

Protein fingerprinting can be used on serum samples to help accurately distinguish between cancer of the prostate, benign prostate hyperplasia (BPH) and healthy tissue. Proteins are detected by a protein-chip array and an artificial intelligence learning algorithm is used to reduce the number of proteins found down to the number that are required to differentiate prostate cancer from noncancer cohorts. 2DGE and mass spectrometry have been used to study serum proteins expressed in patients with BPH and those with high-grade prostatic intraepithelial neoplasm (Gu et al. 2008). Serum amyloid A was found to be expressed in the cancer patients, but weakly or not at all in those with BPH.

Biomarkers, such as autoantibody signatures, may improve the early detection of prostate cancer. Autoantibodies against peptides derived from prostate-cancer tissue could be used as the basis for a screening test for prostate cancer. Fetuin-A, an established tumor antigen in several types of cancer, has been implicated in prostate cancer and autoantibodies specific for fetuin-A show usefulness as a prognostic indicator for prostate cancer patients prone to progress to metastatic disease (Mintz et al. 2015).

Serum-fingerprinting method has a higher specificity than PSA test for differentiating prostate cancer from BPH and unaffected healthy men. This approach can substantially reduce unnecessary prostate biopsies. Another advantage of this technique is that prostate cancer might be detected earlier than with PSA screening. The next step is to identify other biomarkers that can differentiate aggressive cancers from nonaggressive cancers, to make this classification system for early detection as effective as possible.

Concluding Remarks on Biomarkers of Prostate Cancer

Despite many promising candidates, no single biomarker has satisfied the criteria as the ideal biomarker. Limited clinical use of IL-6, TGF-β1 and PCA3 has started, and further widespread availability of these tests is expected in the near future. The trend is to use artificial neural networks and panels of biomarkers instead of individual assays. Although PSA has some well-known limitations, it remains the best biomarker available for prostate cancer when used in conjunction with nomograms or risk calculators.

There is a tremendous need for better prognostic biomarkers in prostate cancer to assist in the identification of patients with aggressive forms of the disease who can potentially benefit from earlier and more intensive forms of treatment. Potential biomarkers of prostatic cancer include caveolin-1, p-Akt, p27, the met oncogene, Ki67 (MIB-1), 8q24 over-expression, polycomb protein EZH2, plasma TGF-B1 and IL-6 among others.

Renal Cancer Biomarkers

Renal cell carcinoma (RCC) is a form of kidney cancer that involves malignant transformation of cells of the renal tube. It is the most common type of kidney cancer in adults. RCC accounts for ~3% of adult malignancies and 90–95% of neoplasms arising from the kidney. In the US >32,000 new cases of RCC are diagnosed every year, and ~12,000 persons die from the disease annually. RCC metastasizes easily, often spreading to the lungs and other organs. In cases where metastatic disease is not yet present at time of diagnosis, the 5-year survival rate for RCC patients is 60–75%. However, metastases are already present at diagnosis in nearly one-third of RCC cases. In cases where the tumor has metastasized to the lymph nodes, the 5-year survival rate is reduced to 5–15% and further to <5% when the cancer has spread to other organs.

Gene Expression Profile of RCC for Biomarkers

The WHO system defines histopathologic tumor subtypes of RCC with distinct clinical behavior and underlying genetic mutations. In adults, the common malignant subtypes are variants of RCC. RCC has a poor prognosis and unpredictable course and to date there are no molecular markers that reliably predict RCC outcome. Histopathologic classification of RCC is critical for clinical management of RCC, but is becoming more complex with recognition of novel tumor subtypes, development of procedures yielding small diagnostic biopsies, and emergence of molecular therapies directed at tumor gene activity. Therefore, classification systems based on gene expression are likely to become essential for diagnosis, prognosis and treatment of kidney tumors. DNA microarray studies have shown that clinically relevant renal tumor subtypes are characterized by distinct gene expression profiles, which are useful for discovery of novel diagnostic and prognostic biomarkers.

miRNA Biomarkers of Renal Cancer

miRNA microarray analysis for comparison of miRNA expression levels between RCC tissues and their normal counterpart revealed several dysregulated miRNAs, which were validated by quantitative RT-PCR and bioinformatics analysis (Chow

et al. 2010). Some of these miRNAs are dysregulated in other malignancies as well and have a potential role in RCC pathogenesis. The miRNAs showed a significant correlation with reported chromosomal aberration sites. Target prediction algorithms were to identify gene targets. These miRNAs are potential biomarkers of RCC. A study has demonstrated the usefulness of miRNA expression profiling for identifying a signature unique to various RCC subtypes at a single anatomic locus (Petillo et al. 2009).

Use of Proteomics for Detection of RCC Biomarkers

Quantitative MS analysis has been used to identify proteins that are dysregulated in RCC (Siu et al. 2009). Protein expression of kidney cancer tissues was compared to their normal counterparts from the same patient using LC-MS/MS. iTRAQ labeling that enabled simultaneous quantitative analysis. These dysregulated proteins in RCC were statistically significantly different from those of transitional cell carcinoma and end-stage glomerulonephritis. These results were validated using different tools and databases including Serial Analysis of Gene Expression (SAGE), UniGene EST ProfileViewer, Cancer Genome Anatomy Project, and Gene Ontology consortium analysis.

Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) has been identified as a protein that is released from mitochondria in response to apoptotic stimuli and promotes apoptosis by antagonizing inhibitor of apoptosis proteins. Detection and quantification of Smac/DIABLO activity (i.e. protein transcription and expression) in cancer tissue following biopsy or surgery may be accomplished through IHC, RT-PCR, Western blot, flow cytometry, and/or HPLC.

Multivariate analyses using proteomic data obtained by SELDI-MS have been reported to be highly successful for detection of various tumors by examination of serum samples. CE-MS has been used to analyze urine samples from patients with RCC and 86 peptides were found to be specifically associated to RCC, of which sequence could be obtained for 40 (Frantzi et al. 2014). A classifier based on these peptides was evaluated in an independent set of samples and results showed 80% sensitivity and 87% specificity. Thus RCC can be detected with a high degree of accuracy based on specific urinary peptides.

Use of RCC Biomarkers for Prognosis and Therapy

RCC responds poorly to chemotherapy and radiation therapy. Cytotoxic chemotherapy, an integral therapeutic component for solid and non-solid tumors, shows little or no antitumor activity against RCC. Complete eradication of RCC by chemotherapy is therefore unlikely, unless all of the cancer can be removed by surgery. Radiation therapy is usually unsuccessful in treating RCC and is therefore not commonly used. In light of the fact that chemotherapy and radiation therapy

mediate their anti-tumor effects by inducing apoptosis, poor response of RCC to these conventional therapies has been hypothesized to associate with the signal transduction pathway that mediates apoptosis in RCC. Two key pathways have recently gained particular attention: the hypoxia response pathway associated with te von Hippel-Landau (VHL) tumor suppressor gene and mTOR (mammalian target of rapamycin) signaling pathway. RCC is associated with loss of functional VHL protein (pVHL) and high, homogeneous expression of the G250MN protein, with a close correlation between hypoxia-inducible function (HIF)-1 expression and G250MN. Inherited mutation of the VHL gene is a strong risk factor for RCC and somatic mutation of VHL gene is one of the most frequent genetic changes seen in RCC.

A novel kidney cancer biomarker, transmembrane carbonic anhydrase IX (CAIX), has been investigated as an independent prognostic factor for survival for patients with metastatic RCC. CAIX plays an essential role in allowing cancer cells to buffer their intracellular pH in hypoxia and/or in intensive glycolysis conditions. CAIX proteins may have a role in the regulation of cell proliferation in response to HIF and may be involved in tumor progression. In patients with non-metastatic RCC low CAIX predicts a worse outcome similar to patients with metastatic disease and overall CAIX expression decreases with development of metastasis. CAIX reflects significant changes in tumor biology, which may be used to predict clinical outcome and identify high-risk patients for adjuvant-targeted therapies. So far the overall evidence points to a connection between VHL mutation and CAIX expression that can be used as a prognostic marker to predict treatment outcome in patients with RCC. Further studies are needed to determine the molecular pathways involved in such tumors as this would have important therapeutic implications.

Expression level, transcription regulation, and biological activity of Smac/DIABLO determine in large part the pathogenesis of RCC and the response of RCC to antitumor therapies including surgery, chemotherapy, irradiation, immunotherapy, gene therapy, toxins and antibodies. The relative gene expression was analyzed by real-time RT-PCR in tumor tissue obtained from patients following surgical treatment and was correlated to clinico-pathological variables and outcome (Kempkensteffen et al. 2008). Expression of Smac/DIABLO is inversely associated with outcome of RCC patients and lack of its expression in RCC predicts a worse prognosis. In addition, transfection with Smac/DIABLO sensitizes RCC to TRAIL/cisplatin-induced apoptosis. Thus Smac/DIABLO expression in RCC may be used as a prognostic parameter, and enhancement of Smac/DIABLO expression in RCC may potentiate immunotherapy and chemotherapy. Analysis of Smac/DIABLO expression will be of significant clinical importance in the design of therapeutic strategies. Smac/DIABLO expression can be induced by use of agents that sensitize cells to apoptosis (e.g. chemicals, inhibitors, biologicals, antisense RNA, gene transfection reagents). An alternative strategy would be to use protease inhibitors to prevent degradation of Smac/DIABLO. Enhanced expression or reduced degradation resulting from these therapeutic approaches may cause the spontaneous induction of apoptosis, or may be used synergistically in combination with low-dose chemotherapy, radiation, or immunotherapy.

Preoperative neutrophil to lymphocyte ratio (NLR) may serve as a potential prognostic biomarker in patients with sarcomatoid RCC (sRCC) and help with clinical decisions about treatment in clinical practice (Gu et al. 2016). A nomogram based on elevated NLR, derived NLR and platelet to lymphocyte ratio can be used for the prediction of overall survival in patients with sRCC.

Thyroid cancer Biomarkers

Approximately 4% to 7% of the general population develops a clinically significant thyroid nodule during their lifetime. The incidence of papillary thyroid carcinoma (PTC) in women is growing faster than any other type of malignancy. In many cases, preoperative diagnoses by fine needle biopsy are inconclusive. Although fine needle aspiration cytology is very useful in the diagnosis of PTC, its accuracy and utility would be greatly facilitated by the development of specific biomarkers for PTC and its common variants. Prognostic value of plasma calcitonin and CEA doubling-time and the presence of somatic RET mutations in MTC tissue, may be useful tools in clinical decision making (van Veelen et al. 2009).

Detection of BRAF Mutation

Many effective methods are available to detect BRAF mutation in FNB material. Because of its high specificity, this genetic alteration is now considered a useful diagnostic marker for patients who have indeterminate thyroid nodule cytology and is a useful tool for thyroid nodule management despite its low sensitivity limiting its application. In future, the screening of genetic alterations will enter standard clinical practice as an adjunctive tool to conventional cytology, and larger studies will provide a better definition of the best, most cost-effective combinations of markers and methods (Marotta et al. 2011).

Gene Expression Biomarkers of Thyroid Cancer

Candidate biomarkers of thyroid cancer include well known genes such as MET, TFF3, SERPINA1, TIMP1, FN1, and TPO as well as relatively novel or uncharacterized genes such as TGFA, QPCT, CRABP1, FCGBP, EPS8 and PROS1. An initial microarray study using the ThermoFisher Scientific's 1700 Chemiluminescent Microarray Analyzer was performed on surgically resected thyroid lesions to identify molecular signatures that distinguish PTC from benign tissue and to discriminate between common variants of PTC (Finn et al. 2007). Selected targets were validated using TaqMan® Real-Time PCR. The data generated corroborate previously identified potential biomarkers as LGALS3, S100A11, LYN, BAX, and CD44. However, the study highlighted numerous transcripts never previously

implicated in thyroid carcinogenesis (many of which are not represented on other microarray platforms). Diminished expression of metallothioneins featured strongly, suggesting a possible role as PTC tumor suppressors. Fifteen transcripts were significantly associated with follicular variant PTC and genes in this subcategory were associated with a narrow repertoire of functions, including MHC and cathepsin families.

In another study, differentially expressed genes (DEGs) were identified using the Linear Models for Microarray Analysis package, and subsequently, common DEGs were screened for functional and pathway enrichment analysis using the Database for Annotation Visualization and Integrated Discovery (Qu et al. 2016). Two of the identified DEGs, FN1 and SERPINA1, may be potential biomarkers for PCT by regulating epithelial-to-mesenchymal transition and responding to steroid hormone stimuli, respectively. This study identified ocriplasmin, β-mercaptoethanol and recombinant α 1-antitrypsin as potential drugs for the treatment of PTC.

miRNA Biomarkers of Thyroid Cancer

miRNA expression profiling correlates with various cancers, with these genes thought to act as both tumor suppressors and oncogenes. ret/PTC 1 is an oncogene with constitutive kinase activity implicated in the development of PTC. This rearrangement leads to aberrant MAPK activation that is implicated in PTC tumorigenesis. As miRNAs are stable, abundant, and very easily detectable, they may be ideal candidates for diagnostic biomarkers. They are also resilient and detectable in archival material.

A serum miRNA profiling study on patients with thyroid masses revealed differences in miRNA levels between benign nodules and control, and between PTC and control with were statistically significant fold changes in the level of 4 miRNAs between benign and PTC: hsa-miR-146a-5p and hsa-miR-199b-3p were down-regulated, while hsa-let7b-5p and hsa-miR-10a-5p were up-regulated (Graham et al. 2015). Thus, serum miRNA has the potential to aid in diagnosis of PTC. Exploration on a larger cohort of samples will hopefully consolidate the correlation between these miRNAs and their host genes in PTC and help identify miRNA biomarkers.

Multiple Endocrine Neoplasia Type 2B as Risk Factor for Thyroid Cancer

Multiple endocrine neoplasia type 2B (MEN2B) is an autosomal dominant, inherited cancer syndrome. MEN2B patients have a high risk of developing medullary thyroid carcinoma (MTC), and prophylactic thyroidectomy is recommended by 6 months of age. Genetic testing can identify MEN2B patients before cancer progression. Two RET proto-oncogene mutations, in exon 15 at codon 883 and in exon 16 at codon 918, account for more than 98% of MEN2B cases. An assay using unlabeled probes and the LightCycler 480 instrument was developed to genotype

these two common MEN2B RET mutations (Margraf et al. 2008). This is a rapid, closed-tube method that is less time consuming and less expensive than sequencing. This assay demonstrates 100% specificity and sensitivity for the identification of RET mutations causative of MEN2B.

MTC shares biochemical features with other neuroendocrine tumors but some particular characteristics could be used as biomarkers. Biochemical studies of histologically proven MTC show that plasma dopamine is increased in the majority of the patients with stable disease and progressive disease, but it does not correlate with extent of disease. Elevated plasma platelet levels of serotonin are only present in patients with MEN2 with stable disease or progressive disease but do not differ between those groups. In addition to plasma calcitonin, only carcinoembryonic antigen (CEA) and chromogranin A can differentiate between stable and progressive MTC. MTCs are capable of synthesizing catecholamines, serotonin, and histamine metabolites indicating metabolic characteristics in common with other neuroendocrine tumors but clinical usefulness and relevance of these findings is limited.

Survivin and XIAP, inhibitors of apoptosis proteins (IAPs), demonstrate distinct expression patterns in MTCs, which are associated with advanced disease and poor prognosis (Werner et al. 2016). Both IAPs might serve as viable targets in patients with MTC.

Role of the NCI in Cancer Biomarkers

The National Cancer Institute (NCI), part of the NIH, is the Federal Government's principal agency for cancer research and training. Activities of NCI are shown on website: https://www.nih.gov/about-nih/what-we-do/nih-almanac/national-cancer-institute-nci.

NCI's Cancer Biomarkers Research Group promotes research to identify, develop, and validate biological markers for early cancer detection and cancer risk assessment. Activities include development and validation of promising cancer biomarkers, collaborative databases and informatics systems, and new technologies or the refinement of existing technologies. Biomarker development should follow an orderly process wherein one proceeds to the next phase only after meeting prespecified criteria for the current phase. Various phases are as follows:

- Phase 1 refers to preclinical exploratory studies, and is usually characterized by ranking and selection, or finding suitable ways to combine biomarkers.
- Phase II has two important components: (1) upon successful completion of the phase I requirements, an assay is established with a clear intended clinical use; and (2) the assay is evaluated for its clinical performance in terms of 'sensitivity' and 'specificity' with thresholds determined by the intended clinical use.
- Phase III involves evaluation of the sensitivity and specificity of the test for the detection of diseases that have yet to be detected clinically. The specimens analyzed

in this evaluation phase are taken from study subjects before the onset of clinical symptoms, with active follow-up to ascertain disease occurrence. Most biomarker validation studies end with this phase and the biomarker will be ready for clinical use.

- Phase IV evaluates the sensitivity and specificity of the test on a prospective cohort. It can estimate the false referral rate based on tested biomarkers and describe the extent and characteristic of disease detected, eg, tumor stage at the time of detection.
- Phase V evaluates the overall risks/benefits of the new diagnostic test on the screened population. The cost per life saved is one example of an endpoint for such study.

A major program of the group is the Early Detection Research Network (EDRN) – a collaborative network that maintains comprehensive infrastructure and resources critical to the discovery, development and validation of biomarkers for cancer risk and early detection. The program comprises a public/private sector consortium to accelerate the development of biomarkers that will change medical practice, ensure data reproducibility, and adapt to the changing landscape of biomarker science.

PSA is an example of a test that did not go through phases IV and V before its widespread clinical use for prostate cancer screening and its clinical benefits are still under debate. Validation of biomarkers for cancer based on NCI-EDRN is shown in Fig. 13.2.

Future Prospects for Cancer Biomarkers

Cancer Biomarker Research at Academic Institutions

In addition to the important role played by the NCI, several academic institutions are actively engaged in cancer biomarker development projects. This supplements the considerable activity in this area in the commercial sector. Critical Path Institute (Tucson, Arizona), an independent third party, has a cancer biosignature program. It persuaded several pharmaceutical companies to share their methods to determine if EGFR is a valuable biomarker and which test is best for predicting efficacy of anticancer drugs targeting it. Specific collaborative initiatives include nanotechnology development; identification of new bloodborne biomarkers for early detection of ovarian, colorectal, lung, and breast cancers; discovery of new drug targets for advanced stages of these cancers; and discovery of new drug targets for childhood cancers. The agreement represents a continuation of a NCI project to develop proteomics technologies for analysis of cancer and other diseases.

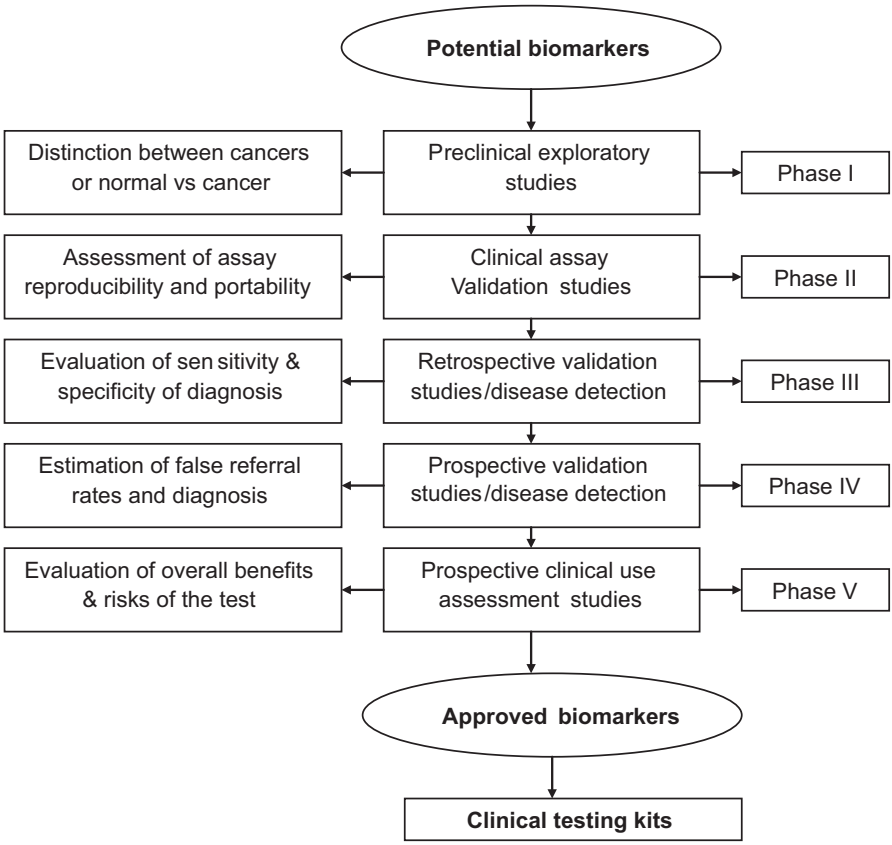

Fig. 13.2 Cancer biomarker development and validation. (© Jain PharmaBiotech)

Future Challenges in the Discovery of Cancer Biomarkers

There is intense activity in identifying novel biomarkers for cancer, especially those for early detection, and already too many biomarkers have been described in the literature. Because there is no central repository of data pertaining to any cancer, it is difficult to estimate if we have too many proteins described as potential biomarkers for any cancer. Technical advances in genomics, transcriptomics, and proteomics have facilitated high-throughput studies in which the data are analyzed in isolation and a comparison with the published literature is not generally possible for the entire dataset. A central repository will not only integrate all the information scattered across the literature, but will also serve as a reference for prioritizing and systematic testing of candidate biomarkers.

Out of the thousands of biomarkers for cancer that have been discovered, only a few are approved by the FDA. With the advent of new and improved genomic and

proteomic technologies such as DNA and tissue microarray, 2D GE, MS and protein assays coupled with advanced bioinformatic tools, it is possible to develop biomarkers that are able to reliably and accurately predict outcomes during cancer treatment. In the future, a serum or urine test for every phase of cancer may guide clinical decision making, supplementing or replacing currently existing invasive techniques. A major challenge will be the integration of proteomics with genomics and metabolomics data and their functional interpretation in conjunction with clinical results and epidemiology. Several genes are up- and down-regulated in cancer, making it problematic to rely on any single tumor biomarker even for one type of cancer, whereas the microenvironment of most of tumors, such as hypoxia and acidosis are hallmarks of tumors at both very early and advanced stages of development. They are promising biomarkers for tumor targeting.

Considerable scientific effort has been made in the past to find common SNPs that correlate with risk of cancer. Protein biomarkers are now considered to be more effective in risk assessment, early detection, and cancer prevention. However, proteomics researchers have not discovered enough cancer-related biomarkers and this is where the bottleneck lies. There is need for accelerating research efforts if protein biomarker identification and validation are to have an impact in battling the disease. The discovery phase for biomarkers needs to be ramped up and multiplexed. Whereas traditional drug discovery is limited to administering just one therapeutic in a patient or model organism at a time, a single serum or tissue sample from a patient can serve as a test for thousands of biomarkers at once. Multiplexing in this way could make trials much more high-throughput and accelerate protein biomarker discovery. There is a need for efficient, low-cost assays to develop and validate candidate biomarkers. ELISA tests remain very expensive and time-consuming; scientists need highly sensitive assays that are far less costly and do not require two antibodies for each protein to develop them.

Targeted therapeutics has provided challenges for imaging techniques to assess tumor response to treatment because many new agents cause cytostasis rather than cytotoxicity. Advanced tracer development, image acquisition, and image analysis have been used to produce quantitative biomarkers of pathophysiology, with particular focus on measurement of tumor vascular characteristics. There is a need for developing comprehensive compound-specific imaging biomarkers that are appropriate for every class of targeted therapeutics.

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Chapter 14
Biomarkers of Disorders of the Nervous System

Introduction

In spite of all the advances in neurology, particularly in the last decade of the twentieth century (Decade of the Brain), there are serious deficiencies in our understanding of the pathomechanism of several neurological disorders as well as our ability to diagnose and treat these disorders. Biotechnologies are being increasingly applied in neurology to address some of these deficiencies (Jain 2013). Novel biomarker identification for neurological disorders will address the current shortcomings in their diagnosis and therapeutics. Basic challenges to biomarker identification in neurological disease are:

- Limited availability of tissue from the site of pathology.
- Paucity of biomarkers of neurological disorders in blood, urine and saliva.
- CSF, the main source of biomarkers in CNS disorders, requires a lumbar puncture.
- Poor clinical diagnostics and extent of disease progression at the time of presentation.
- The complexity of the brain and tissue heterogeneity.
- The lack of functional endpoints and models for validation.

Discovery of Biomarkers for Neurological Disorders

Figure 14.1 shows discovery and application of biomarkers in neurological diseases.

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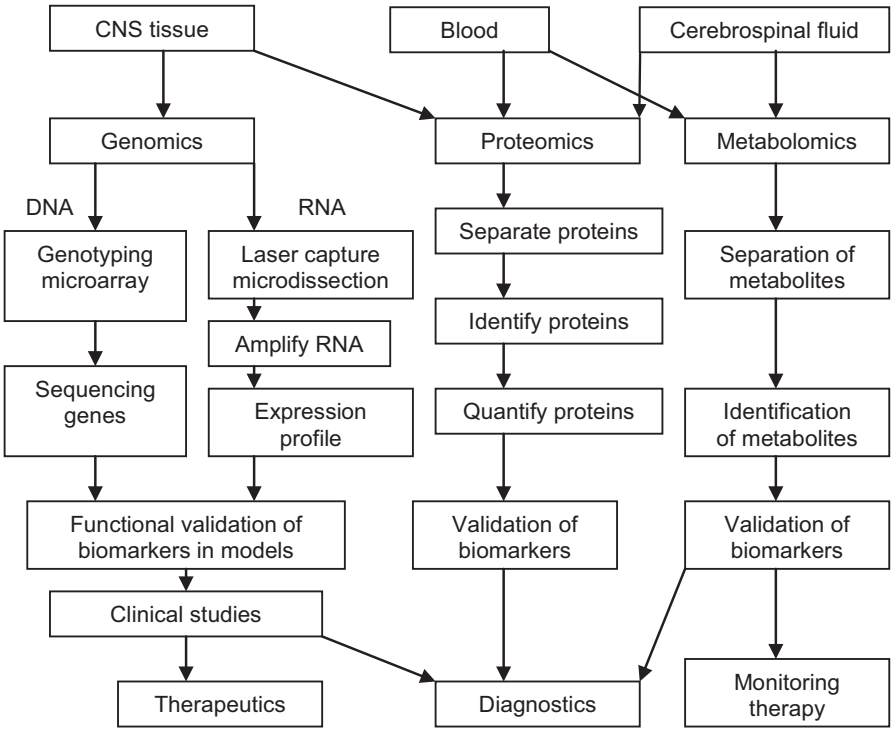

Fig. 14.1 Discovery and application of biomarkers in neurological diseases (© Jain PharmaBiotech)

Desirable characteristics of a biomarker vary according to the disease. General characteristics of an ideal biomarker of CNS disease are:

- It should be noninvasively (or minimally invasively) detectable in living subjects
- Results should be reproducible
- It should be positively correlated to the cause or progression of the disease

Compared with acute neurological conditions, biomarker discovery in chronic progressive and relapsing neurodegenerative diseases may be more difficult because the degenerative process may be so slow that the biomarker discovered is released only in small quantities and it may be difficult to differentiate acute new damage, relevant to prognostic estimates, from already existing background damage. However, a potential advantage in chronic conditions is that there may be sufficient time for the disease to leave a specific signature on the biomarker by the means of post-translational modifications. Hypothesis-driven biomarker discovery, which focuses on post-translational modifications such as phosphorylation, aggregate for-

mation or changes in protein stoichiometry, may open new avenues for successful biomarker discovery in chronic and relapsing conditions such as dementia and multiple sclerosis (Kuhle and Petzold 2011).

Plasma or serum are the most convenient source of biomarkers. However, a pathological process in the CNS is not always reflected in the systemic compartments, and the detection of such biomarkers has been mostly limited to neurological diseases that have an autoimmune or metabolic basis. Refined proteomic technologies are now being used to detect biomarkers of neurodegenerative disorders in the blood.

Biomarker Identification in the CSF Using Proteomics

Cerebrospinal fluid (CSF) is an important source of potential biomarkers for neurologic disorders. CSF is also a rich source of biomarkers of systemic disorders such as peptides, and antibodies that are capable of crossing the blood-brain barrier (BBB). Proteomic technologies such as immunoblotting, isoelectric focusing, 2DGE and MS have proven useful for deciphering the CSF proteome. CSF proteins are generally less abundant than their corresponding serum counterparts, necessitating the development and use of sensitive analytical techniques. Brain extracellular fluid (ECF), mostly obtained by cerebral microdialysis and subjected to proteomic analysis along with CSF, is also a good source of biomarkers of CNS disorders.

A tandem mass tag approach, consisting of a set of structurally identical tags, which label peptides on free amino-terminus and epsilon-amino functions of lysine residues, has been for quantitative MS-based proteomics of CSF (Dayon et al. 2008). Human postmortem CSF was taken as a model of massive brain injury and comparison was carried out with antemortem CSF. Peptides were identified and quantified by MS/MS analysis with MALDI TOF/TOF and ESI-Q-TOF. The concentration of 78 identified proteins was shown to be clearly increased in postmortem CSF samples compared to antemortem. Some of these proteins, like GFAP, protein S100B, and PARK7, are already known as biomarkers of brain damage, supporting the use of postmortem CSF as a valid model of brain insult. ELISA for these proteins confirmed their elevated concentration in postmortem CSF.

Biomarker Identification in the CSF Using Lipidomics

Lipids comprise the bulk of the dry mass of the brain. In addition to providing structural integrity to membranes, insulation to cells and acting as a source of energy, lipids can be rapidly converted to mediators of inflammation or to signaling molecules that control molecular and cellular events in the brain. ESI and atmospheric pressure chemical ionization have enabled compositional studies of the diverse lipid

structures that are present in brain. These include phospholipids, ceramides, sphingomyelin, cerebrosides, cholesterol and their oxidized derivatives. Lipid analyses have delineated metabolic defects in neurological disorders. In this review, we examine the structure of the major lipid classes in the brain, describe methods used for their characterization, and evaluate their role in neurological diseases. Protein biomarkers in the CSF may be potential therapeutic targets since they transport lipids required for neuronal growth or convert lipids into molecules that control brain physiology. Combining lipidomics and proteomics will enhance existing knowledge of disease pathology and increase the likelihood of discovering specific markers and biochemical mechanisms of brain diseases.

Cerebral Microdialysis for the Study of Biomarkers of Cerebral Metabolism

In the past, the neurochemistry of the brain was primarily evaluated indirectly via samples of the CSF, measurements of cerebral metabolism or the evaluation of spectra from nuclear magnetic resonance studies. In order to get direct estimates of the neurochemical concentrations of the tissue, brain samples had to be collected and assayed – a rather impractical approach for in vivo studies. Microdialysis offered a unique opportunity to explore human brain neurochemistry without the need for tissue extraction. Neurochemical biomarkers for cerebral metabolism that can be monitored by cerebral microdialysis are shown in Table 14.1.

Table 14.1 Biomarkers of cerebral metabolism

Neurochemical process	Biomarkers
Disturbed glucose metabolism	Glucose/lactate, pyruvate, lactate/pyruvate, pH
Excitotoxicity	Glutamate (aspartate)
Increased adenosine triphosphate (ATP) utilization	Adenosine, inosine, hypoxanthine
ATP depletion	K^+, neurotransmitter release
Cellular membrane degradation	Glycerol
Reactive oxygen species formation	Xanthine, urate, allantoin, ascorbate, glutathione, cysteine, spin-trap metabolites
Nitric oxide formation	Nitrite, nitrate, citrulline/arginine
Neurotransmitter release	GABA, glycine, noradrenaline, dopamine, serotonin
Ionic perturbations	Na+, Ca^{2+}, Mg^{2+}
Neuroinflammation	IL-1, IL-6, GFAP, NGF
Blood-brain barrier leakage	Alanine, valine, leucine

Detection of Protein Biomarkers of CNS Disorders in the Blood

Antibody-based tests can measure proteins in the blood. Various biomarkers found in blood include S100 protein, neuron-specific enolase, myelin basic protein and C-tau. These will be described under various neurological disorders later in this chapter.

Concentrations of the S100 protein, an acidic calcium-binding protein found in the gray matter of the brain, are elevated in serum after brain damage. Several commercial ELISA assays are available for S100 protein and are useful biochemical markers for the early assessment of brain damage by the quantitative determination of S100 in serum.

Genomic Technologies for Study of Biomarkers of Neurological Disorders

Due to the complex nature of neurological disorders, it is difficult to identify the mechanisms using conventional methods, where only small pathways around specific target genes are investigated. The advent of systems biology approaches has made it possible to study these complex problems from the whole-genome perspective. Genomic technologies have been increasingly applied to the investigation of neurological disorders and involve the investigation of the genome, transcriptome and epigenome. There are two types of technologies available for genomic studies, including sequencing and various array platforms. For the investigation of genomic variation, the samples generally come from peripheral blood although saliva has also been used. For the investigation of transcriptome, brain tissue is the most studied since it is more relevant to the disease mechanism. The peripheral blood and CSF have also been investigated, mostly for the discovery of novel biomarkers. These three tissues have also been utilized in the investigation of epigenomic alteration. In addition, skin fibroblast has been increasingly used in induced pluripotent stem cell (iPSC) technologies.

Brain Imaging for Detection of Biomarkers

Imaging techniques enable the diagnosis of disease in vivo. Several specialized techniques have evolved during the past quarter of a century for imaging pathology in the living brain. These include CT, MRI, PET and SPECT (see Chap. 2).

MRI sensors that directly and rapidly respond to chemicals involved in the brain's information processing would provide a much more precise measurement of brain activity. A MRI sensor that responds to the neurotransmitter dopamine may significantly improve the specificity and resolution of brain imaging procedures

(Shapiro et al. 2010). The sensor is derived from the heme domain of the bacterial cytochrome P450-BM3. This new tool connects molecular phenomena in the nervous system with whole-brain imaging techniques, enabling precise exploration of processes and relating them to the overall function of the brain. The protein engineering approach can be generalized to create probes for other targets. Molecular fMRI reveals much more specific information about the brain's activity and circuitry than conventional blood-related fMRI.

Although imaging biomarkers are used in diagnostic workup of neurological patients, the use in clinical trials has been limited. Changes in brain pathology, visualized by imaging, can be used as an in vivo guide to the treatment. Brain imaging has an important role to play in detection of biomarkers of neurodegenerative diseases and these will be described later in this chapter.

Brain imaging, mainly ultrasound and MRI, is useful as a biomarker of brain injury in the newborn due to hypoxia-ischemia, brain hemorrhage or infection. However, the challenge is to correlate brain structure with function, and new technologies will provide insights into the function of the developing brain (Austin and O'Reilly 2011). Imaging will play a key role as an early biomarker to facilitate clinical trials of neuroprotective therapies.

Diffusion tensor imaging (DTI) tractography is a biomarker and early endpoint in traumatic brain injury (TBI) and aneurysmal subarachnoid hemorrhage (aSAH). DTI parameters, assessed at approximately day 12 after injury, correlate with mortality at 6 m in patients with severe TBI or aSAH with similar patterns for both (Sener et al. 2016).

Biomarkers of the Aging Brain

Cellular Biomarker of Aging of the Brain

Impairment of memory occurs with aging. A cellular biomarker, which plays a critical role in regulating neuronal excitability, has been identified in the hippocampus and can be used to understand and identify the mechanisms of aging of the brain (Oh and Disterhoff 2010). It is determined by integrated electromyography recordings of the eyeblink responses during delay and trace eyeblink conditioning from a young adult rat.

CSF F2-Isoprostanes as Biomarker of Aging Brain

Free radical injury has been implicated in the pathogenesis of AD, but it also is associated with aging brain. Free radical injury in the CNS was quantified with CSF F2-isoprostanes (IsoPs) measurements in clinically normal individuals and a

significant increase was observed over the adult human lifespan (Montine et al. 2011). Relationship between age and CSF F2-IsoPs was compared for clinically normal adults with normal CSF and those with abnormal CSF Aβ42 and/or tau indicating AD; only those with normal CSF demonstrated a significant increase with age. These results show that CSF F2-IsoPs increased across the human lifespan and that this age-related increase in free radical injury to brain persisted after excluding those with laboratory evidence of latent AD.

IL-6 as a Biomarker of Cognitive Impairment with Aging

A study has investigated the associations between both high concentration of IL-6 and the −174 promoter polymorphism, and increased cognitive decline in old age (Mooijaart et al. 2013). Over 5000 participants of the Prospective Study of Pravastatin in the Elderly at Risk (PROSPER) with a mean age of 75 years and a history of cardiovascular disease or its risk factors were included in the present study. The investigators determined baseline concentrations of IL-6 and genotype of the IL6–174 polymorphism, of which the C allele was previously shown to be associated with higher circulating concentrations of IL-6. A cognitive test battery was administered at baseline and repeatedly during follow-up (mean 39 months). Cross-sectional analysis of participants showed that higher IL-6 concentration was associated with worse executive cognitive function, independent of cardiovascular disease status. No association was found between IL-6 concentration and memory function. In the prospective analysis, higher IL-6 concentration was associated with an increased rate of cognitive decline in both executive function and memory function, again independent of cardiovascular disease status and risk factors. Although not associated with IL-6 concentrations, the IL6-174 CC genotype was associated with worse performance on the Stroop test. The study concluded that higher circulating levels of IL-6 are associated with worse cognitive function and steeper cognitive decline, and provide preliminary genetic evidence for a potential causal association. The findings support the use of high IL-6 levels a biomarker to predict cognitive decline.

Protein Aggregation as a Biomarker of Aging Brain

In neurodegenerative diseases, such as Alzheimer disease (AD) and Huntington disease (HD), specific proteins escape the cell's quality-control system and associate together, forming insoluble aggregates. A study, conducted in *C. elegans*, has shown that widespread protein insolubility and aggregation is an inherent part of aging, and it may influence both lifespan and neurodegenerative diseases (David et al. 2010). There are ~700 proteins in a normal *C. elegans* that become insoluble with age. Proteins similar to those aggregating in old worms have also been identified as

minor components of human disease aggregates. In the presence of proteins specific to HD actually sped up the course of the disease. About half of the aggregating proteins in AD become insoluble as a normal part of aging. The protein aggregation was significantly delayed or even halted by reducing insulin and IGF-1 hormone activity, which is known to extend animal lifespan and to delay the progression of HD and AD in animal models. The scientists also found that gene manipulations that extend the lifespan of *C. elegans* prevented the formation of these insoluble aggregates. Inherent protein aggregation is a new biomarker of aging. Understanding how to modulate it will lead to important insights into the mechanisms that underlie aging and protein aggregation diseases.

Telomere Shortening as a Biomarker of Aging Brain and Dementia

Telomeres are protective DNA sequences located at the ends of chromosomes that shorten with each division of the cell. Although telomeres are known biomarkers of cellular aging, their association with whole organism aging and various diseases is currently being explored. A study has revealed an association between longer telomeres obtained from leukocytes of peripheral blood and a decreased risk of mortality or dementia over a median period of 9.3 years (Honig et al. 2012). It is not certain if short telomeres are mere biomarkers of aging rather than a determinant of the aging process. If telomere length turns out to be a determinant of aging, therapies directed at modifying telomere length shortening by increasing telomerase activity may be helpful in decreasing the incidence of age-related dementia.

Data Mining for Biomarkers of Neurological Disorders

The Stanley Medical Research Institute (http://www.stanleyresearch.org/) online genomics database (SMRIDB) is a comprehensive data mining tool to enable researchers to elucidate the biological mechanisms of bipolar disorder, schizophrenia, and depression. A diverse patient population combined with data generated across six microarray platforms and various studies to provide robust results to enhance the understanding of brain disease. It offers researchers an efficient tool for data mining of brain disease complete with information such as: crossplatform comparisons and biomarkers elucidation for target discovery.

Weighted correlation network analysis (WGCNA) can be used for finding clusters (modules) of highly correlated genes (Langfelder and Horvath 2008). Correlation networks facilitate network based gene screening methods that can be used to identify candidate biomarkers or therapeutic targets. The WGCNA package is freely available at http://www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork/Rpackages/WGCNA.

Antibodies as Biomarkers in Disorders of the Nervous System

The autoantibodies are routinely tested in many neuropathies, neuromuscular junction disorders, and myopathies and can be very useful biomarkers for diagnosis and management. However, in CNS disorders, the pathogenic role of autoantibodies is still emerging. There is growing evidence for predominant antibody mediated demyelination in a subgroup of multiple sclerosis patients who may possibly be identified by presence of autoantibodies. This will be discussed in more detail later in this chapter. The autoantibodies in the peripheral nervous system can help detect a specific tumor and are routine tested in suspected cases of paraneoplastic syndromes. The early detection and treatment of underlying tumor in peripheral nervous system may lead to clinical improvement in some cases. Clinical response to plasma exchange and intravenous immunoglobulin in certain CNS disorders associated with anti-glutamic acid decarboxylase, GluR3, voltage-gated calcium channel, and voltage-gated potassium channel antibodies provides indirect evidence for a pathogenic role of these autoantibodies.

In autoimmune encephalitis, differences have been described between limbic encephalitis associated with antibodies targeting intracellular antigens, and neuronal surface antibody syndromes (NSAS) where the antigens are primarily receptors or synaptic proteins located on the neuronal cell surface (Ramanathan et al. 2014). Developments in NSAS, particularly in voltage gated K channel complex-associated antibody mediated encephalitis, anti-N-methyl-d-aspartate receptor encephalitis, and anti-dopamine 2 receptor antibody-associated basal ganglia encephalitis, show the complexities of using serum antibodies as biomarkers.

The search for more autoantibodies and the effort to better define their contribution to the disease process are ongoing. In the future, new paradigms for their detection and treatment of antibody mediated diseases may be established.

Biomarkers of Neural Regeneration

Biomarkers are useful for assessment of regeneration in the nervous system and also for assessment of effect of agents such as nerve growth factor (NGF) that promote regeneration. One way to do this is to measure neurite outgrowth, is a slow and tedious process. There is little work done on this important topic although more information is available about biomarkers that impede regeneration such as soluble Nogo-1.

Surrogate biomarkers for neurite outgrowth activity by gene expression analysis have been identified in SH-O10 cells, subclones of the human SH-SY5Y neuroblastoma cell line, which have a much higher NGF-induced neurite outgrowth activity (Oe et al. 2006). Microarray analysis revealed seven genes where mRNA levels were changed. NGF-induced decreases in levels of two genes, CyclinB2 and BIRC5, were confirmed by quantitative real-time RT-PCR. Decreases in CyclinB2 and

BIRC5 mRNA induced by FK506 or retinoic acid, both of which enhance NGF-induced neurite outgrowth effects, correlated with their neurite outgrowth activities. They concluded that decreasing levels of CyclinB2 and BIRC5 mRNA strongly correlate with neurite outgrowth activities in terms of NGF-related effect in SH-SY5Y subclonal cells, and have potential to become quantitative surrogate biomarkers for measuring NGF-related neurite outgrowth.

Later studies on regeneration following CNS trauma have led the identification of exosomes, nano-sized extracellular vesicles, which have key roles in cell signaling, and might serve as novel biomarkers as well as vehicles for targeted delivery of repair-inducing molecules (Werner and Stevens 2015).

Biomarkers of Disruption of Blood-Brain Barrier

Blood-brain barrier (BBB) is a dynamic conduit for transport between blood and brain of those nutrients, peptides, proteins, or immune cells that have access to certain transport systems localized within the BBB membranes. Recent advances in cell and molecular biology have provided new insights into the function of the BBB. Several disorders of the central nervous system (CNS) are associated with increased permeability of the BBB. Two main approaches are used for studying the integrity of human BBB in vivo: (1) structural imaging employs contrast agents that only penetrate the BBB at sites of damage, and (2) functional imaging is used to study the transport of substances across the BBB – both intact and damaged. Structural imaging employs contrast agents with CT scanning and is relatively insensitive. MRI with the contrast agent gadolinium is more sensitive. Functional imaging is done with PET and can quantify cerebral uptake of therapeutic agents, such as cytotoxic agents and monoclonal antibodies. SPECT is less versatile than PET, but can provide semiquantitative measurement of BBB leakage of albumin or red blood cells. There is a need for biomarkers to detect early changes in BBB.

Loss of integrity of the BBB resulting from ischemia/reperfusion is believed to be a precursor to hemorrhagic transformation (HT) and poor outcome in acute stroke patients. An MRI biomarker has been used to characterize early BBB disruption in human focal brain ischemia and its association with reperfusion, HT, and poor outcome. Reperfusion was found to be the most powerful independent predictor of early BBB disruption and thus of HT and is important for the decision for acute thrombolytic therapy. Early BBB disruption as defined by this imaging biomarker is a promising target for adjunctive therapy to reduce the complications associated with thrombolytic therapy, broaden the therapeutic window, and improve clinical outcome in acute stroke.

The astrocytic protein S100B is a potentially useful peripheral biomarker of BBB permeability. Other biomarkers of BBB have been discovered by proteomic approaches. These proteins are virtually absent in normal blood, appear in serum from patients with cerebral lesions, and can be easily detected by commercially available ELISA tests. S100B levels in peripheral blood are raised in

soldiers under stress leading to an increase BBB permeability secondary to immune activation, which is associated with stress-related depression and anxiety (Li et al. 2014a).

Biomarkers of Neurotoxicity

Neurotoxicity may be defined as any adverse effect on the structure or function of the central and/or peripheral nervous system by a biological, chemical, or physical agent. A multidisciplinary approach is necessary to assess adult and developmental neurotoxicity due to the complex and diverse functions of the nervous system. The overall strategy for understanding developmental neurotoxicity is based on two assumptions: (1) significant differences in the adult versus the developing nervous system susceptibility to neurotoxicity exist and they are often developmental stage dependent; (2) a multidisciplinary approach using neurobiological, including gene expression assays, neurophysiological, neuropathological, and behavioral function is necessary for a precise assessment of neurotoxicity. Application of genomic approaches to developmental studies must use the same criteria for evaluating microarray studies as those in adults including consideration of reproducibility, statistical analysis, homogenous cell populations, and confirmation with non-array methods. Studies using amphetamine to induce neurotoxicity supports the following: (1) gene expression data can help define neurotoxic mechanism(s), (2) gene expression changes can be useful biomarkers of effect, and (3) the site-selective nature of gene expression in the nervous system may mandate assessment of selective cell populations. Desirable features of biomarkers of neurotoxicity are:

- Indicate respond to diverse types of insults affecting any region of the brain
- Sensitive with low incidence of false-negatives
- Specific to the neurotoxic condition with low incidence of false-positives
- Simple to evaluate
- Quantitative

Glial Fibrillary Acidic Protein as Biomarker of Neurotoxicity

The glial reaction, gliosis, represents a hallmark of all types of nervous system injury. Therefore biomarkers of gliosis can be applied for assessment of neurotoxicity. The astroglial protein, glial fibrillary acidic protein (GFAP), can serve as one such biomarker of neurotoxicity in response to a panel of known neurotoxic agents. Qualitative and quantitative analysis of GFAP has shown this biomarker to be a sensitive and specific indicator of the neurotoxicity. The implementation of GFAP and related glial biomarkers in neurotoxicity screens may serve as the basis for further development of molecular signatures predictive of adverse effects on the nervous system.

Single-Stranded DNA as a Biomarker of Neuronal Apoptosis

Single-stranded DNA (ssDNA) is a biomarker of apoptosis and programmed cell death, which appears prior to DNA fragmentation during delayed neuronal death. A study investigated the immunohistochemical distribution of ssDNA in the brain to investigate apoptotic neuronal damage with regard to the cause of death in medicolegal autopsy cases (Michiue et al. 2008). Neuronal immunopositivity for ssDNA was globally detected in the brain, independent of the age, gender of subjects and postmortem interval, and depended on the cause of death. Higher positivity was typically found in the pallidum for delayed brain injury death and fatal CO intoxication, and in the cerebral cortex, pallidum and substantia nigra for drug intoxication. For mechanical asphyxiation, a high positivity was detected in the cerebral cortex and pallidum, while the positivity was low in the substantia nigra. The neuronal ssDNA increased during the survival period within about 24 h at each site, depending on the type of brain injury, and in the substantia nigra for other blunt injuries. The neuronal positivity was usually lower for drowning and acute ischemic disease. Topographical analysis of ssDNA-positive neurons may contribute to investigating the cause of brain damage and survival period after a fatal insult.

Biomarkers of Neurogenetic Disorders

A neurogenetic disorder is defined as a disease caused by a defect in 1 or more genes that affect the differentiation and function of neuroectoderm and its derivatives. There are 2 types of neurogenetic disorders. Type 1 neurogenetic disorders include those resulting from malfunction of genes expressed in the neuroectoderm. Most of the classic inherited disorders belong to this category. Type 2 neurogenetic disorders are those in which neurologic manifestations are caused indirectly by the abnormal function of a gene not expressed in the nervous system. Type 2 includes metabolic diseases with neurologic manifestations as well as cerebrovascular and cranial malformations. Some of the neurodegenerative disorders described in the following section also have a genetic component.

Both environmental factors and genetic predisposition contribute to cause birth defects. Congenital malformations of the nervous system frequently arise sporadically, making it difficult to determine whether or not they are genetic in origin, let alone which gene or genes may be involved. Rapid progress has been made during recent years in the localization and identification of gene mutations in specific malformations. However, an individual carrying the disease-related gene may never develop the manifestations of the disease. Adult-onset genetic disorders usually arise as a result of disturbances of enzyme metabolism, slow accumulation of toxic substances, slow tissue death, or inability to repair DNA damage. With new molecular genetics techniques an understanding of the molecular pathology of the disease is not essential for diagnostic or predictive tests.

Charcot-Marie Tooth Disease

The genes for this disease have been mapped to chromosome 17 (Charcot-Marie Tooth 1A), chromosome 7 (Charcot-Marie Tooth X), and another unknown chromosome (Charcot-Marie Tooth 1C). It has been found that 70% to 80% of patients with a clinical diagnosis of Charcot-Marie-Tooth 1 carry the 17p11.2–12 duplication, implying that an assay for duplication provides a powerful marker for screening suspected patients and family members at risk. Other forms of Charcot-Marie-Tooth are associated with mutations in the myelin protein Z (Charcot-Marie Tooth 1B) and Cx32 (Charcot-Marie Tooth X) genes. Thus, mutations in different genes can cause similar Charcot-Marie-Tooth phenotypes. Mutations in the peripheral myelin protein 22 and myelin protein Z genes can also cause the related but more severe neuropathy, Dejerine-Sottas syndrome. All genes so far identified by Charcot-Marie-Tooth investigators appear to play an important role in myelin formation or maintenance of peripheral nerves.

Conventional disease detection depends on electrodiagnostic tests including EMG and nerve conduction velocity measurements. The isolation of genes underlying these conditions has facilitated both the differential and molecular diagnosis of these disorders by single-stranded conformation polymorphism, direct DNA sequencing, or both. Other methods used to detect CMT1A gene duplication are pulsed field gel electrophoresis, restriction fragment length polymorphism, and fluorescence in situ hybridization. Another approach is the use of Southern blot and amplification by polymerase chain reaction of polymorphic poly repeats (microsatellites) located within the duplicated region, or the detection of junction fragments specific for the duplication. A pulsed field gel electrophoresis-based CMT1A DNA test is available. It detects CMT1A duplication in 70% to 90% of the patients. These methods require radioactive markers or other complicated procedures and are time-consuming and labor-intensive. A PCR-based test provides results within 24 h for detection of a recombinant hotspot associated with CMT1A duplication. One diagnostic strategy uses highly polymorphic short tandem repeats located inside the CMT1A duplicated region. Combined use of the three short tandem repeats enables robust diagnosis with almost complete certainty. As DNA testing for Charcot-Marie-Tooth 1A becomes more widely available, it may become an accepted part of the evaluation of any patient with a suspected hereditary neuropathy.

Increased levels of PMP22 in compact myelin of peripheral nerves have been demonstrated and presumed to cause the phenotype of CMT1A. The extra copy of PMP22 in CMT1A results in disruption of the tightly regulated expression of PMP22. Thus, variability of PMP22 levels, rather than absolute level of PMP22, may play an important role in the pathogenesis of CMT1A (Katona et al. 2009).

Duchenne and Becker Muscular Dystrophy

There are over 40 primary congenital muscle disorders as determined by the defective genes causing the disorders rather than specific clinical descriptions. The most common form of muscular dystrophy is X-linked Duchenne muscular dystrophy (DMD) and the milder form called Becker muscular dystrophy (BMD) is the second most common form of the disease. Together DMD and BMD affect approximately 1 in 3600 newborn males per year worldwide and comprise nearly 80% of all new cases of dystrophy and 56% of all new cases of congenital myopathy of all types. The underlying cause of DMD and BMD is abnormalities of the DMD gene, encoding the protein called dystrophin.

The location of the dystrophin gene is on chromosome Xp21. This has been confirmed by DNA polymorphism linking. This is the largest gene known in the human genome, spanning 2.5 megabases. Most DMD cases are caused by out-of-frame mutations in the dystrophin gene followed by absence of dystrophin. In contrast, most BMD cases result from in-frame mutations that allow the expression of truncated partially active protein.

The first molecular test for DMD, Chamberlain-Beggs Multiplex PCR, detected 98% of large deletions and some duplications in the dystrophin gene. Sometimes when the test detected one or more deleted or duplicated exons, additional adjacent exons not in the hot spot region had to be tested separately in order to find the break points which are important for differentiating BMD from DMD. This test has now been replaced in most laboratories by the Multiplex Ligation-Dependent Probe Amplification (MLPA) test for aberrant copy number in the DMD gene. MLPA tests all 79 exons in two reactions, and detects the exact molecular cause, including break points, for approximately 60% of DMD and BMD cases. Various forms of direct sequencing and exon pre-screening from patient DNA have been used to detect the majority of the remaining 40% of mutations in the DMD gene. These remaining mutations are mostly small (less than one exon) variations collectively known as point mutations including insertions, duplications, deletions, deletions plus insertions (indels) and single or multiple base changes. One of the most common and efficient methods for detecting mutations in the exons and regions of interest in disease genes is PCR amplification of the area of interest resulting in millions of copies of that DNA fragment followed by direct sequencing of the fragment. An automated process using direct sequencing following PCR has been developed for the detection of deletions, duplications/insertions and point mutations in any gene or family of genes and has been applied to ten genes known to bear mutations that cause muscular dystrophy (Bennett et al. 2009).

Among non-PCR methods, an immunoblot assay can detect abnormalities in dystrophin in the absence of detectable PCR deletions. DMD-specific FISH probes are useful for the detection of carriers of DMD gene deletions.

Metabolite ratios as measured by in vivo proton magnetic resonance spectroscopy and muscle function scores are significantly decreased in patients with DMD when compared with normal control subjects. A statistically significant decrease in

trimethyl ammonium/total creatine ratio in patients with DMD, as compared with control subjects, was found to correlate with decreased muscle function (Hsieh et al. 2009). miR-206 and other muscle-specific miRNAs in serum are useful for monitoring pathological progression DMD (Hu et al. 2014). Rise of serum levels of matrix metalloproteinase-9 (MMP-9) indicate pathological progression of DMD (Nadarajah et al. 2011).

Early-Onset Torsion Dystonia

Early-onset torsion dystonia (EOTD) is characterized by involuntary and sustained muscle contractions that can lead to paralysis and abnormal posture. It is known that patients with EOTD have a mutated gene (glutamate ΔE) that encodes the protein TorsionA, but the effect of ΔE on torsinA and the reason that this mutation results in EOTD were unclear. Moreover, there are no specific treatment of EOTD. A yeast torsinA expression system was developed to test the roles of ER chaperones in mediating the folding and stability of torsinA and torsinAΔE (Zacchi et al. 2014). The authors of this study discovered that, BiP (binding immunoglobulin protein), an endoplasmic reticulum lumenal Hsp70 and Lhs1 (a nucleotide exchange factor) stabilize torsinA and torsinAΔE. BiP also maintained torsinA and torsinAΔE solubility. Mutations predicted to compromise specific torsinA functional motifs showed a synthetic interaction with the ΔE mutation and destabilized torsinAΔE, suggesting that the ΔE mutation predisposes torsinA to defects in the presence of secondary insults. In this case, BiP was required for torsinAΔE degradation, consistent with data that specific chaperones exhibit either pro-degradative or pro-folding activities. Finally, it was shown that BiP stabilizes torsinA and torsinAΔE in mammalian cells. Together, these data define BiP as the first identified torsinA chaperone, and can be considered a biomarker of EOTD. Treatments that modulate BiP might improve symptoms associated with EOTD by affecting TorsinA.

Fragile X Syndrome

Molecular diagnosis of fragile X syndrome (FXS) has been developed using Southern hybridization methods or PCR approaches. Cytogenetics, which has been the major diagnostic tool used for FXS until the discovery of the FMR1 gene, is no longer required to establish the diagnosis but is still considered to be a supplementary test to rule out any other chromosomal abnormalities in a child with mental retardation. DNA testing is considered to be more sensitive than cytogenetic analysis (100% versus 50%) and more cost effective. One drawback of DNA-based diagnosis is that IQ scores cannot be predicted, and for this the emerging technique of FMR1 protein quantification in target tissues should be considered.

One approach to prenatal diagnosis of FXS is an antibody test on amniotic fluid cells. Simple PCR combined with blood spot analysis could be a reliable, inexpensive test that is feasible for a large-scale screening of male subjects with mental retardation for FXS but Southern blot assay with mixed DNA is appropriate for screening female subjects. There is delayed early-phase phosphorylation of extracellular-signal regulated kinase (ERK), a nodal point for cell signaling cascades, in both neurons and thymocytes of fmr-1 KO mouse model of FXS. Early-phase kinetics of ERK activation in lymphocytes from human peripheral blood is delayed in a cohort of individuals with FXS, relative to normlal controls, suggesting a potential biomarker to measure metabolic status of disease for individuals with FXS (Weng et al. 2008).

Genetic Neurotransmitter Disorders

The monoamine neurotransmitter disorders are a heterogeneous group of neurogenetic disorders involving defects in the metabolism of dopamine, norepinephrine, epinephrine and serotonin. The inheritance of these disorders is mostly autosomal recessive. The neurological symptoms are primarily due to cerebral deficiency of dopamine, serotonin or both. The clinical presentations are highly variable and substantial overlaps exist. Accurate diagnosis requires laboratory investigations. Measurement of neurotransmitter metabolites in the CSF is the key to identification of metabolic defects; other investigations including plasma phenylalanine, urine pterins, urine 3-O-methyldopa and serum prolactin are helpful in establishing the diagnosis (Siu 2015). Genetic analyses are essential for confirming the diagnosis to enable specific treatments, proper genetic counselling, prediction of prognosis, assessment of risk of recurrence in the family as well as prenatal diagnosis. Early diagnosis with appropriate treatment is associated with remarkable response and favorable clinical outcome in several disorders in this group.

Hereditary Neuropathy with Liability to Pressure Palsies

Hereditary neuropathy with liability to pressure palsies (HNPP) is a dominantly inherited disorder that presents as recurrent mononeuropathies precipitated by trivial trauma. HNPP has been attributed to a 1.5 mb deletion in 17p11.2 spanning the peripheral myelin protein 22 (pmp22) gene. Underexpression of the pmp22 gene causes hereditary neuropathy with liability to pressure palsies just as overexpression of PNP22 causes Charcot-Marie Tooth 1A. Thus, 2 different phenotypes can be caused by dosage variations of the same gene.

Analysis of DNA can be used to detect the clinically unaffected members of families. Genetic biomarker screening is an efficient diagnostic strategy; in about

90% of cases, which reveals the presence or absence of 17p deletion in HNPP. Families with HNPP have been identified that lack the 17p deletion and possess 2 normal copies of PNP22 gene, and it is possible that mutations are present in other parts of the gene that have not yet been examined. One study found that PMP22 levels were reduced in peripheral nerve myelin in dermal skin biopsies in HNPP patients with an Leu7fs mutation (Li et al. 2007). Clinical and electrophysiological evaluation showed that patients with the Leu7fs mutation were indistinguishable from patients with HNPP caused by chromosome 17p11.2 deletion, confirming that the phenotypic expression is identical in both types of patients and that reduction of PMP22 is sufficient to cause the full HNPP phenotype.

Hereditary Metabolic Storage Disorders with Neurologic Manifestations

Several hereditary metabolic disorders can be diagnosed by laboratory methods of detecting biochemical disturbances. Genes for some of these disorders have been localized, and molecular diagnostic methods have been used for the detection of these diseases as shown in the following examples.

Gaucher Disease

Gaucher disease is an inherited lysosomal storage disorder, characterized by massive accumulation of glucosylceramide-laden macrophages in the spleen, liver and bone marrow as a consequence of deficient activity of glucocerebrosidase. Molecular diagnosis of Gaucher disease is complicated by the presence of a pseudogene near the true gene on chromosome 1 q21. Selective amplification of the true gene is possible by duplex-PCR test, which involves amplification of the genomic DNA and hybridization to oligonucleotide probes. Signals are detected by chemiluminescence.

Several new therapeutic interventions such as enzyme-replacement therapy and substrate-reduction therapy have been developed for this condition. The availability of these costly therapies has stimulated research regarding suitable biomarkers to monitor onset and progression of disease, as well as the efficacy of therapeutic intervention. Given the important role of storage cells in the pathology, various attempts have been made to identify proteins in plasma or serum reflecting the body burden of these pathological cells (Boot et al. 2009). Two of these biomarkers are worth noting: (1) marked increase of plasma chitotriosiderase activity has been validated as a biomarker of Gaucher's disease; and (2) overproduction of plasma chemokine PARC/CCL18 is a viable alternative biomarker and is particularly useful in patients who are chitotriosiderase deficient.

Pompe's Disease

Pompe disease is characterized by acid maltase deficiency and is considered to be type II glycogen storage disease. With regard to enzymatic analysis, the application of acarbose as inhibitor of maltase-glucoamylase has enabled the use of mixed leukocyte preparations as diagnostic material. The use of glycogen as a natural substrate in the reaction mixture adds to the selectivity of this procedure. Newborn screening is envisaged and facilitated by the introduction of high-throughput procedures. DNA analysis has become an integral part of the diagnostic procedure for confirmation and completion, for carrier detection, and for genetic counseling (Reuser et al. 2010).

Mitochondrial Disorders Affecting the Nervous System

Mitochondria generate energy for cellular processes by producing adenosine triphosphate through oxidative phosphorylation. These organelles contain their own extrachromosomal DNA, which is distinct from DNA in the nucleus. Diseases, particularly those affecting organs with high-energy requirements such as the brain and the muscles, have been linked to defects in the mtDNA. Features that suggest a mitochondrial origin for disease are maternal inheritance and defect in mitochondrial oxidative phosphorylation. There is increasing acceptance of the role of defective mitochondrial energy production and the resulting increased level of free radical production in the pathogenesis of various neurodegenerative disorders such as Huntington disease, Parkinson disease, and amyotrophic lateral sclerosis. These defects may contribute to both excitotoxic and oxidative damage. The evidence comes from a similarity to known mitochondrial disorders including delayed and variable age of onset, slow progression, and symmetric degeneration of localized groups of neurons.

A complete human mtDNA sequence is available and a set of sensitive and specific molecular genetic tests has been developed for a number of mitochondrial diseases. In suspected mitochondrial disease, a small portion of the muscle biopsy is frozen in liquid nitrogen for DNA isolation. Because most mtDNA mutations occur in relatively few families, it is important to do a comprehensive analysis of mtDNA by single-strand conformation polymorphism and sequencing to rule out a mtDNA mutation as a cause of symptoms in an individual. The diagnosis is based on integrated clinical genetic and biochemical information. The unequivocal establishment of the diagnosis of mitochondrial disease by mtDNA examination is a prerequisite for proper genetic counseling and, eventually, for treatment. Transmitochondrial cybrids can be used to test the effects of either mitochondrial or nuclear gene abnormalities in a fully controlled, user friendly, and highly informative system.

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA), which occurs approximately 1 in 6000 live births, is associated with a mutation in chromosome 5, and is among the most common genetic causes of infantile death. Muscle weakness, lack of motor development, and poor muscle tone are the major clinical manifestations of SMA in infants. For the most severe form of the disease, the lifespan of patients is expected to be ~2–3 years, and others may live later into childhood, or into early adulthood.

The gene for SMA is called survival motor neuron (SMN) gene. Localization of the major genes for SMA has led to prenatal detection using chorionic villous or amniotic fluid cells. With the identification of the survival motor neuron gene, direct DNA analysis of amniotic cells is now possible. In fact, no false-negative predictions to date have been recorded. However, false-positive predictions (deletion detected in the absence of clinical disease) have been reported, suggesting that the exact role of the SMN gene is not clear. Carriers can be detected using a quantitative PCR or a fluorescence-based technique. Most SMA carriers have only one copy of SMN1. However, about 5% of expected carriers have two copies of SMN1 on 1 chromosome and none on the second chromosome. Therefore, routine carrier testing is not necessarily specific.

Biomarkers of SMA

SMA presents challenges in (i) monitoring disease activity and predicting progression, (ii) designing trials that allow rapid assessment of candidate therapies, and (iii) understanding molecular causes and consequences of the disease. Validated biomarkers of SMA motor and non-motor function would offer utility in addressing these challenges. Drug targets for SMA have been discovered, but clinical development has been limited partly by a need for qualified biomarkers of disease progression or of disease amelioration. In 2011, NINDS funded research to evaluate biomarkers and clinical outcomes longitudinally and across the spectrum of SMA cases. Another aim was to establish a clinical outcomes and biomarker database, and it contributed samples to the NINDS biorepository at the Coriell Institute for Medical Research.

Spinal Muscular Atrophy Foundation, New York, conducted a study with the objectives: (i) to discover additional markers from the Biomarkers for SMA (BforSMA) study using an immunoassay platform; and (ii) to validate the putative biomarkers in an independent cohort of SMA patients collected from a multi-site natural history study (Kobayashi et al. 2013). Discovery and validation using independent cohorts yielded a set of SMA biomarkers significantly associated with motor function and other measures of SMA disease activity. A commercial SMA-MAP biomarker panel was generated for further testing in other

SMA collections and interventional trials. Future work includes evaluating the panel in other neuromuscular diseases, for pharmacodynamic responsiveness to experimental SMA therapies, and for predicting functional changes over time in SMA patients.

Biomarkers of Neurodegenerative Disorders

Neurodegenerative diseases constitute a large group of diseases of which the best known are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). Current diagnostic methods are helpful in detecting these diseases when CNS damage has already occurred. It is important to detect these diseases at very early stages to improve the therapeutic prospects. Biomarkers-based detection methods are expected to enable early diagnosis and also provide an insight into pathomechanisms of these diseases. Some of the biomarkers may become the bases for development of therapeutics.

Loss of cortical neurons is a key pathological feature in neurodegenerative dementias. CSF neurofilaments (Nf) are a biomarker for neuronal death and axonal loss. Currently, CSF NfH and NfL levels are not recommended for use as a screening test in the diagnosis of dementia because of the rather small effect size. However, both neurofilament proteins may be of value for targeted investigation of some patients with frontotemporal lobe dementia, vascular dementia and AD (Petzold et al. 2007).

Application of quantitative 2DGE, combined with appropriate single-variable and multivariate biostatistics, enables selection of disease-specific serum biomarkers in neurodegenerative disorders. However, there are no proteomic biomarkers of neurodegenerative diseases available in the market, which can fulfill the clinical criteria for specificity and sensitivity for these diseases. Large cohorts of specifically targeted patient blood serum samples and complimentary age-matched controls, in parallel with the use of selected panels of these biomarkers, are being applied to the development of blood tests to specifically address unmet pressing needs in the differential diagnosis of these diseases, and to provide potential avenues for mechanism-based drug targeting and treatment monitoring. Study of protein aggregation in the aging brain is relevant to detection of protein biomarkers of neurodegenerative disorders.

Next generation sequencing also has an impact on neurodegenerative disease research as it improves our understanding of the pathomechanism of various diseases. New gene mutations that are being discovered contribute to a person's risk of disease. Biomarker-based tests would be more practical for diagnosis of neurodegenerative diseases.

Biomarkers of Dementia

Dementia is defined as "a global impairment of higher cortical functions, including memory, the capacity to solve the problems of everyday living, the performance of learned perceptual motor skills and the correct use of social skills and the control of emotional reactions, in the absence of gross clouding of consciousness" (Royal College of Physicians 1981). There are numerous causes of primary dementia include degenerative diseases such as Alzheimer diseases (AD) and chronic cerebrovascular insufficieny such as vascular dementia. There mixed vascular and degenerative dementas as well. Secondary dementias may occur due to other disorders such as metabolic encephalopathies, nutritional deficiencies, neurotoxicity, CNS infections, brain tumors, and normal pressure hydrocephalus. The diagnosis can be established by biomarkers of the primary disease. Biomarkers of neurodegenerative disorders will be described in the following sections.

Biomarkers of Vascular Dementia

There is no general agreement as to its definition of vascular dementia (VD). The term "vascular" may be obsolete and "dementia" implies that a patient has reached a state from which recovery is unlikely but cognitive impairment due to disturbances of blood circulation may be reversible. Alternative term of vascular cognitive impairment has been used. Another term that has been used in the literature in the past is multi-infarct dementia but VD has a broader connotation and is the most widely used term to describe cognitive impairment after stroke. Post-stroke dementia is another term used to describe this condition. The only catch to this is that a patient with vascular disease may develop dementia without any manifest stroke but rather silent strokes. VD is characterized by abnormalities of white matter, which are clearly detectable with CT or MRI. The association of dementia, hypertension, extensive white matter lesions and small subcortical infarcts is characteristic. Subcortical vascular cognitive impairment is now recognized to be the commonest form of vascular cognitive impairment, its clinical pattern, risk factors, and imaging features being sufficiently consistent for it to be considered an entity for the purposes of diagnosis, clinical trials, and management. It is recognized as subcortical vascular dementia in the International Classification of Diseases. Both stroke and AD are common disorders of aging and are commonly associated. It is often difficult to prove whether stroke directly causes VD, contributes to its development or is merely coincidental. Pathologically subcortical VD may be difficult to distinguish from AD in elderly subjects because mixed forms of both dementias exist quite frequently in the same subject.

Cerebral endothelial dysfunction occurs in several neurodegenerative diseases. A population-based prospective study has shown that elevated plasma concentration of midregional pro-adrenomedullin (MR-proADM) is an independent predictor of vascular dementia, and an increase in C-terminal endothelin-1 (CT-proET-1) indicates higher risk of VD as well as other dementia subtypes (Holm et al. 2017).

Biomarkers of Alzheimer's Disease

Biomarkers under investigation for AD are described in detail in a special report on this topic (Jain 2017). Most of these studies are investigating the pathophysiology of the disease. The goals of discovery of new biomarkers for AD are:

- Differential diagnosis of AD
- To follow the efficacy of a new treatment
- To identify new therapeutic targets

Table 14.2 shows a classification of currently known biomarkers of AD in serum and CSF.

Table 14.2 Classification of biomarkers of Alzheimer disease in blood and CSF

Pathophysiology	Biomarkers in CSF		Biomarkers in blood	
Indicators of inflammatory process	α-ACT (antichymotrypsin)	+	α-ACT	+
	Gp130	=	CD11b	+
	Hp (haptoglobin)	=	Hp	=
	Hp fragments	+	Hp 2-1	+
	IL (interleukin)-1β	+	IL-1β	+
	IL-1β receptor	+	IL-6	+
	IL-6	+	IL-6 receptor	–
	IL-6 receptor	=	TNF-α	+
	TNF (tumor necrosis factor)-α	+		
	TNF-α receptor	=		
Indicators of oxidative stress	8-hydroxy-2′-deoxyguanosine	+	8-12-iso-iPF2α-VI	+
	F2-isoprostanes	+	F2-isoprostanes	=
	Neuroprostanes	+		
	3-nitrotyrosine	+		
Vitamins	Vitamin C	=	Homocysteine	+/=
	Vitamin E	–	Vitamin B6	–
			Vitamin B12	–/=
			Folic acid	–
			Vitamin A	–
			Vitamin C	–
			Vitamin E	–

(continued)

Table 14.2 (continued)

Pathophysiology	Biomarkers in CSF		Biomarkers in blood	
Proteins and peptides	Aβ	–	Aβ	+
	ACAT-1 in blood mononuclear cells	+	APP ratio in platelets	+
	β2 microglobulin	+	ALZAS (**Alz**heimer **As**sociated)	+
	CystatinC	+	GFAP-antibodies	+
	Hyperphosphorylated tau	+	Glutamine synthetase	+
	Combination tau with $A\beta_{1-42}$/ $A\beta_{1-40}$ ratio	+	Glycogen synthase kinase-3	+
	GFAP (glial fibrillary acidic protein)	+	Hemeoxygenase 1	–
	GFAP-antibodies	+	IRP(Iron regulating protein) -2	?
	Laminin	+	Protein kinase C	*
	N(epsilon)(gamma-glutamyl) lysine isodipeptide	+		
	Neurosin	–		
	p-Tau	+	p-Tau	+
	VEGF (vascular endothelial growth factor)	–		
Cholesterol and disturbance of lipid metabolism	Cholesterol	+	Cholesterol	+
	Sulfatide	–		

+ Increased levels in AD patients compared to controls
– Decreased levels in AD patients compared to controls
= No difference in AD patients compared to controls
* Conformational change
? Uncertain

To properly evaluate a biomarker for AD, a proposed metabolite would need to be followed prospectively in sufficiently large sample, preferably composed of subjects who would never get the disease, presymptomatic subjects destined to acquire the disease and subjects who have already shown symptoms of the disease. The diagnostic standard for comparison is the clinical examination in the living patient and neuropathological examination where available. In the Framingham study patients, higher spontaneous production of IL-1 or TNF-α by peripheral blood mononuclear cells was considered to be a biomarker of future risk of AD in older individuals (Tan et al. 2007). These data strengthen the evidence for a pathophysiologic role of inflammation in the development of clinical AD. However, some biochemical abnormalities that are observed in the serum of patients with AD may be significant but do not necessarily have value as biomarkers. For example, the plasma amino acid profiles of elderly patients with MCI or AD show abnormalities in aromatic and basic amino acids that potentially affect neurotransmitter biosynthesis. The two AD processes: formation of amyloid plaques and the aggregation of tau are

linked through the enzyme glycogen synthase kinase-3 (GSK-3). The measurement of GSK-3 in circulating white cells from a blood sample is a useful early diagnostic marker for AD. GSK-3 was also found to be elevated in patients with MCI. Several studies have demonstrated altered levels of cytokines in plasma and differential gene expression and protein phosphorylation in peripheral blood mononuclear cells from AD patients. Identification of the roles of these proteins may provide valuable insights into the underlying molecular pathology of AD and possible sites for therapeutic intervention (Pelech 2008).

A study found high neutral lipid levels and increased ACAT-1 (acyl-coenzyme A:cholesterol acyltransferase) protein in 85% of peripheral blood mononuclear cells from patients with probable sporadic AD compared to 7% of cognitively normal age-matched controls (Pani et al. 2009). A significant reduction in high density lipoprotein-cholesterol levels in plasma from AD blood samples was also observed. Additionally, correlation analyses revealed a negative correlation between high density lipoprotein-cholesterol and cognitive capacity, as determined by Mini Mental State Examination, as well as between high density lipoprotein-cholesterol and neutral lipid accumulation. These findings suggest that neutral lipid-peripheral blood mononuclear cells and plasma high density lipoprotein-cholesterol determinations might outline a distinctive metabolic profile in both AD patients and asymptomatic subjects at higher risk of disease.

The Ideal Biomarker for AD

The ideal biomarker for AD was defined as follows by the Consensus Report of the Working Group on Molecular and Biochemical Markers of Alzheimer's disease (Ronald and Nancy Reagan Research Institute and the NIA Working Group-1998) and no significant changes have been made to the criteria. Recommended steps to establish a biomarker include confirmation by at least two independent studies with the results published in peer-reviewed journals. Desirable characteristics of an ideal biomarker for AD are listed in Table 14.3.

A review of current candidate markers indicates that for suspected early-onset familial AD, it is appropriate to search for mutations in the presenilin 1, presenilin 2, and APP genes. Individuals with these mutations typically have increased levels of the Aβ42 peptide in plasma and decreased levels of APPs in CSF. In late-onset and sporadic AD, these measures are not useful, but detecting an ApoeEe4 allele can add confidence to the clinical diagnosis.

Currently no biomarker fulfils the criteria of the ideal biomarker. A combination of Aβ42 and tau from CSF sample may be the best available. If therapies are to be directed against amyloid deposits, we need a marker to tell us if these deposits have started to form. Whether this can be accomplished with any biomarker other than neuroimaging probes remains to be seen.

Table 14.3 Characteristics of an ideal biomarker for Alzheimer disease

It should be directed at the fundamental neuropathology of AD
It should detect the presence of disease at an early asymptomatic stage
It should mark the presence of the disease itself rather than a risk factor
It should track severity of disease pathology at preclinical stage
It should correlate with histological criteria for the diagnosis of the disease
It should have a sensitivity >80% for detecting AD and a specificity of >80% for distinguishing other dementias
It should be reliable and reproducible
The method for detecting the biomarker should be non-invasive
The biomarker should be inexpensive so that it can be repeated at certain intervals
The biomarker should also form the basis of treatment or indicate the efficacy of treatment

Methods for Determining Biomarkers of AD

Gene Expression Patterns in AD

DiaGenic (Oslo, Norway) has developed a test for diagnosis of AD using gene expression patterns in peripheral blood cells based on its patented technology. Positive results were obtained by analyzing blood samples from patients with AD and controls. NEW ADtect® for diagnosis of AD has received CE approval in Europe and will be marketed through a network of distributors. Test analysis will be performed by DNA Vision in Belgium. The disease process starts several decades before the onset of cognitive decline, suggesting that presymptomatic diagnosis of AD and other progressive cognitive disorders may be feasible in the near future. MCItect® (DiaGenic aims to detect AD in MCI patients within 2 years prior to onset of dementia. FDA has cleared this test and a clinical study has been initiated in the USA.

Magnetic Resonance Spectroscopy in AD

Proton MR spectroscopy (MRS) is sensitive to within-individual changes in the concentration of brain metabolites over time. MRS studies have found both decreased N-acetylaspartate (NAA) and increased myo-inositol in the occipital, temporal, parietal, and frontal regions of patients with AD, even at the early stages of the. This diffuse NAA decline is independent of regional atrophy and probably reflects a decrease in neurocellular viability. Reports of such metabolite changes are now emerging in the mild cognitive impairment (MCI) and in investigation of the medial temporal lobe. In vivo quantitation of neural choline in AD has been inconclusive because of poor test-retest repeatability. Less robust evidence using

phosphorous MRS has shown significant phosphocreatine decline and increments in the cell membrane phosphomonoesters in the early, and possibly asymptomatic, stages of the disease. These phosphorous metabolite disturbances normalize with disease progression. Phosphodiester concentration has been found to correlate strongly with AD plaque counts. MRS of AD has therefore introduced new pathophysiologic speculations. Studies of automated MRS for AD diagnosis have reported high sensitivity and moderate specificity, but are yet to test prospective samples and should be extended to include at least two MRS regions of interest. MRS has promise for predicting cognitive status and monitoring pharmacological efficacy, and can assess cortical and subcortical neurochemical change.

Myoinositol, a compound found in the brains of AD patients and those with mild brain problems, can be detected by MRS and may help to identify those at risk of developing AD. Myoinositol is a marker of inflammatory changes in the brain that are part of AD and explain the rise in brain level of this substance. There is an association of metabolic changes in the brain detected by MRS with ApoE genotype and neuropsychological measures of memory and cognition in normally aging elderly, and in patients with mild cognitive impairment (MCI) and AD. Myoinositol (MI)/creatine is a more specific biomarker for neuropsychological dysfunction associated with neurodegenerative disease. However, N-acetylaspartate /MI ratio may be the most efficient predictor of memory and cognitive function in patients with MCI and AD.

Choline therapy should promote decreased cholinergic membrane breakdown and so lead to relative decreases in MRS-visible choline. Choline is significantly decreased in the drug takers versus placebo control subjects. There is greater choline decrement in pharmacological responders versus nonresponders. The combination of therapeutic intervention, repeat MRS, and cognitive assessment is likely to be a useful research design in the investigation of AD.

MicroRNAs as Biomarkers of Neurodegenerative Disorders

Certain microRNAs (miRNAs) are enriched in different brain regions (e.g., hippocampus, midbrain), cells (e.g., neurons), and cellular compartments (e.g., synapses and neurites). miRNAs can cross the BBB and are dysregulated in a number of neurodegenerative diseases. Thus, brain-enriched miRNAs, present in synapses and detectable in plasma, can be effective and patient-friendly biomarkers, reflective of processes underlying brain health conditions. Alteration of miRNA expression have the potential for improving diagnostic tools as well as providing a basis for treatment. Table 14.4 shows differentially expressed miRNAs in some neurodegenerative disorders.

In March 2017, DiamiR LLC received a National Institute on Aging (NIA)/NIH's SBIR phase IIB grant to support further development of the company's targeted diagnostic technology, based on the analysis of brain-enriched miRNA biomarkers in plasma, for detection of AD at presymptomatic, MCI and dementia stages.

Table 14.4 miRNA expression in neurodegenerative diseases

Disease	Gene	miRNA
Huntington's disease	HTT (Huntingtin)	miR-29a, miR-29b-1, miR-132, miR-135b
SCA1	Ataxin	miR-19a, miR-101, miR-130a
SCA3	Ataxin 3	Bantam
Parkinson's disease		miR-133b
Creutzfeld-Jakob disease	PRNP gene	miR-128, miR-139-5p, miR-146a, miR-320, miR-328, miR-337-3p, miR-338-3p, miR-342-3p
Alzheimer's disease		miR-9, miR-15a, miR-19b, miR-22, miR-23b, miR-26b, miR-29a, miR-29b, miR-93, miR-101, miR-103, miR-106a, miR-106b miR-107, miR-125b, miR-138, miR-181c, miR-197, miR-210, miR-219, miR-298, miR-320, miR-328, miR-363, miR-511, mir-520c, miR-574-5p
Amyotrophic lateral sclerosis		miR-9

MRI for Biomarkers of AD

Among the routine imaging studies in neurology, CT does not have much value for diagnosis of AD but MRI is useful for detection of biomarkers. In cognitively normal older adults, biomarkers of neurodegeneration (as reflected by medial temporal lobe atrophy) and of cerebrovascular disease (as reflected by infarcts) as detected by MRI, independently contribute to the risk to develop AD. New developments in molecular imaging involve developing smart molecules that seek out and highlight specific biological processes so they are visible through 3.0 T MRI (GE Global Research). By seeing and analyzing these biological processes, scientists hope to connect certain biomarkers in the brain with the onset of AD. The goal is to develop a viable method for diagnosing AD in patients early enough to enable treatment. The same technology will be used to evaluate the effectiveness of current and future pharmaceutical therapies.

MRI is particularly suitable for detection of brain iron which promotes oxidative damage and protein oligomerization resulting in highly prevalent age-related neurodegenerative disorders such as AD and PD. Previously BBB was considered to be a protection against accumulation of too much iron in the brain from rest of the body. Measurments of amount of iron in ferritin molecules (ferritin iron) with MRI in various brain regions of AD patients in vivo correlate highly with published postmortem brain iron levels. There are significant age-related changes in ferritin iron and it is possible that brain iron accumulation is a risk factor that can be modified. MRI provides the opportunity to assess brain iron levels in vivo and may be useful in targeting individuals or groups for preventive therapeutic interventions.

Functional MRI (fMRI) is a viable technique for identifying persons at risk for AD. fMRI has shown that more areas of differential blood oxygenation level-

dependent response in regions commonly associated with AD pathology in high-risk AD individuals compared with those at lower risk of the disease. Patterns of association between left medial temporal lobe activity and memory performance are also different supporting the theory of upregulation in neuronal memory systems in people at risk for AD many years before the typical age at disease onset. Thus fMRI findings may be used as a potential biomarker for preclinical AD and in clinical trials.

Techniques for visualizing amyloid plaques in living brains, using commonly available brain-scanning technology, would facilitate the development of an early diagnostic test for AD. In APP transgenic mice as models of Aβ amyloidosis, intravenously administered ^{19}F-containing amyloidophilic compound, labels brain plaques and enables them to be visualized in living mice by MRI using ^{19}F and ^{1}H. This provides specific noninvasive amyloid imaging without the danger of exposure to radiation and could be used in longitudinal studies in mouse models of AD to search for biomarkers associated with Aβ pathology as well as to track disease course after treatment with experimental drugs. Much work is still needed before this technology can be used to diagnose AD in humans.

Semiautomated, individually specific quantitative MRI methods can be used to identify a pattern of regional atrophy in MCI that is predictive of clinical decline (McEvoy et al. 2009). Noninvasive neuroimaging MRI biomarkers for AD may enable earlier clinical diagnosis and the monitoring of therapeutic effectiveness (Fennema-Notestine et al. 2009). With mild MCI and AD, greater differences are evident in frontal, temporal, posterior cingulate, inferior parietal, precuneus, and retrosplenial cortices. Subjects with MCI who have phenotypic AD atrophy showed significantly greater 1-year clinical decline and structural loss than those who do not and were more likely to have progression to probable AD (annual progression rate of 29% for subjects with MCI who had AD atrophy vs 8% for those who did not). These findings demonstrate that high-throughput methods provide numerous measures for detection of subtle effects of prodromal AD, suggesting early and later stages of the preclinical state in this cross-sectional sample. These methods will enable a more complete longitudinal characterization and enable identification of changes that are predictive of conversion of MCI to AD. Such information may aid in prediction of patient prognosis and increase the efficiency of clinical trials.

Data of an open study with fMRI monitoring indicate that treatment with galantamine leads to more efficient visual processing of stimuli in the AD patients along the dorsal visual pathway (Bokde et al. 2009). A visual perception task recruiting the dorsal visual system may be useful as a biomarker of treatment effects.

Nanotechnology to Measure Aβ-Derived Diffusible Ligands

Aβ-derived diffusible ligands (ADDLs) comprise the neurotoxic subset of soluble $A\beta_{1–42}$ oligomers, now widely considered to be the molecular cause of memory malfunction and neurodegeneration in AD. Screening based on a fluorescence resonance energy transfer (FRET)-based assay can identify small molecule modulators

of ADDL-mediated neurotoxicity. The identified hits are further characterized by assessing their ability to inhibit the assembly and binding of ADDLs to cultures of primary hippocampal neurons. This approach has led to the identification of a number of small molecules which inhibit ADDL assembly and their subsequent binding to neurons. Some of the validated hits are being advanced into preclinical lead compounds. Acumen Pharmaceuticals Inc. is developing diagnostics that detect serum ADDLs and tagged ADDLs in the brain. ADDLs are soluble and cross the BBB, allowing a serum-based diagnostic assay. A serum ADDL diagnostic will change the way clinical trials for AD and MCI are conducted. By screening patients before, and monitoring their cognitive function during clinical trials, Acumen expects shorter, small clinical trials with remarkably better "signal-to-noise" ratio compared clinical protocols commonly used today for testing promising AD therapies.

Commercial enzyme-linked immunoassays (ELISA) can only detect ADDLs in brain tissue where the biomarker is most highly concentrated. Studies of ADDLs in the CSF have not been possible because of their low concentration. A nanoparticle-based ultrasensitive bio-barcode assays can be used to measure the concentration of ADDLs in CSF. The bio-barcode amplification technology, which is a million times more sensitive than ELISA, can detect ADDLs in the CSF even if they are present in very low concentrations. ADDL concentrations for the subjects diagnosed with AD are consistently higher than the levels in the CSF taken from nondemented age-matched controls. ADDLs should be validated as biomarkers of AD in blood.

PET Scanning for Biomarkers of AD

Fluorine-18 fluorodeoxyglucose PET is used for the evaluation of relative cerebral glucose metabolic rate (rCMRglc). In patients presenting with cognitive symptoms of dementia, regional brain metabolism is a sensitive indicator of AD. In patients with MCI, characteristic cerebral metabolic differences can be delineated at the time of initial presentation, which helps to define prognostic subgroups. A newly emerging reduction of rCMRglc in prefrontal cortical areas is associated with the transition from MCI to AD. 18F–FDG PET of cerebral glucose metabolism is thus a valuable diagnostic tool for the prediction of clinical outcome in individual MCI patients. Results are superior to the exclusive assessment of the APOE genotype. Combination of both functional imaging and genotyping may enable an early high-risk or low-risk stratification of patients with either very high sensitivity or very high specificity. This may be valuable for patient selection in clinical trials. PET is also used for studies of memory function and receptor imaging. It is possible to map cerebral AChE activity using PET. It has a potential application in drug development and in vivo molecular neuropharmacology. It may be possible to use PET for predicting the response of AD patients to cholinergic therapy. Avid Radiopharmaceuticals Inc. is developing novel radiolabeled PET and SPECT compounds that specifically and sensitively bind Aβ for diagnosis and monitoring treatment of AD.

Nicotine α4ß2 receptor subtypes are implicated in the study of AD and smoking addiction. A putative antagonist, nifrolidine, has been evaluated as a PET agent for nicotine α4ß2 receptors. Nifrolidine is a selective binding agent that identifies specific areas of the brain responsible for decision-making, learning and memory. Nifrolidine provides reliable, quantitative information of these receptors and might be very useful for future human PET studies of nicotine addiction and other clinical conditions in which these brain regions have been implicated. Patients with AD have been known to have a loss of nicotine receptors and nifrolidine could be a potential marker for early diagnosis of AD.

Potential applications of in vivo PET imaging of Aβ plaques are as follows:

- Improvements in early diagnosis and the possibility of a presymptomatic diagnostic biomarker.
- Improved understanding of the natural history of amyloid deposition and insights into the pathophysiology of MCI and AD.
- Quantification of amyloid burden to assess the effects of newly developed anti-amyloid therapies such as secretase inhibitors, immunotherapy and plaque breakers.

Abnormal brain protein deposits that define AD can be detected in persons with MCI by use of PET performed after injection of 2-(1-{6-[(2-[F-18]fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene)malononitrile (FDDNP), a molecule that binds to plaques and tangles in vitro. On the basis of cognitive testing, the subjects are classified as having AD or MCI or no cognitive impairment (healthy controls). FDG-PET and MRI are also performed. FDDNP-PET binding differentiates among the diagnostic groups better than metabolism on FDG-PET or volume on MRI. FDG, which measures the metabolic function of cells, cannot identify the abnormal brain protein deposits that may cause the disease. FDDNP-PET scanning can differentiate persons with MCI from those with AD and those with no cognitive impairment. This technique is potentially useful as a noninvasive method to determine regional cerebral patterns of amyloid plaques and tau neurofibrillary tangles. It may also enable testing of novel drug therapies and manage disease progression over time for neuroprotection. A clinical trial is being conducted with this molecular biomarker in order to obtain FDA approval, which would make the method available for use by physicians on their patients.

Florbetapir (Avid Radiopharmaceuticals/subsidiary of Eli Lilly & Co) is used with PET to enable visualization of the Aβ deposits in the brain that are characteristic of AD. It started phase II clinical trials in 2008. Previous PET imaging studies of Aβ plaque have involved the use of ^{11}C-labeled radiopharmaceuticals, which have a very short useful radioactive half-life (20 min), whereas florbetapir may be used over several hours after production due to the longer (2 h) half-life, making it practical for broader scale availability in the future. In a prospective clinical evaluation, florbetapir-PET imaging was correlated with the presence and density of Aβ (Clark et al. 2011). Florbetapir-PET scans identified the characteristic plaques in 97% of patients who actually had them, as determined by a subsequent autopsy. These data

provide evidence that a molecular imaging procedure can identify Aβ pathology in the brains of individuals during life. Florbetapir ^{18}F binds tightly to Aβ plaques making them visible to the scanner but does not bind to other tissue. These scans were negative on young and healthy subjects. It was the first radioactive diagnostic agent for AD approved by the FDA. Additional studies may be required to understand the appropriate use of florbetapir-PET imaging in the clinical diagnosis of AD and for the prediction of progression to dementia.

Florbetaben (Bayer's BAY 94-9172) is a ^{18}F-labeled polyethyleneglycol stilbene derivative with high affinity to synthetic $A\beta_{1–42}$ fibrils and post-mortem human AD brain homogenates. From preliminary data of studies on AD patients using intravenous administration, it was concluded that florbetaben-PET (FBB-PET) has a potential to diagnose AD in the living human brain (Barthel et al. 2008). Phase II and III clinical trials were completed successfully to evaluate the efficacy and safety of FBB-PET imaging in the detection of Aβ plaques in the brain. An open-label, nonrandomized, multicenter, phase III study was done to validate florbetaben by comparing in vivo PET imaging with post-mortem histopathology (Sabri et al. 2015). FBB-PET showed high sensitivity and specificity for the detection of histopathology-confirmed Aβ plaques and may thus be a valuable adjunct to clinical diagnosis, particularly for the exclusion of AD. It was approved by the FDA and EMA. Although FBB-PET has not become a standard procedure in the diagnosis of dementia, it can be a helpful additional diagnostic tool when used according to the "Appropriate Use Criteria" and the S3 guidelines on dementia in cases of unclear clinical presentation, atypically early age of onset as well as in patients with persistent or progressive unexplained MCI (Schönecker et al. 2017). By facilitating early diagnosis it enables patient selection for therapeutic drug trials. Potential applications of in vivo PET imaging of Aβ plaques are:

- Improvements in early diagnosis and use as a presymptomatic diagnostic biomarker.
- Improved understanding of the natural history of amyloid deposition and insights into the pathophysiology of MCI and AD.
- Quantification of amyloid burden to assess the effects of newly developed anti-amyloid therapies such as secretase inhibitors, immunotherapy and plaque breakers.

Simultaneous Measurement of Several Biomarkers for AD

The xMAP technology has been used to develop and evaluate a multiparametric bead-based assay (Innogenetic's INNO-BIA AlzBio3) for quantification of Aβ1-42, total tau (T-TAU), and hyperphosphorylated tau (P-TAU) in CSF. This multianalyte assay format has been compared with established ELISA techniques for the same proteins. A clinical study using CSF samples from patients with AD or MCI with progression to AD, healthy controls, and patients with other neurologic disorders demonstrated that this multiparametric assay could accurately distinguish patients

with AD from patients with other neurologic disorders or control patients, with the diagnostic accuracy reaching recommended consensus criteria for specificity and sensitivity. The new multiparametric method may be able to replace the corresponding ELISA methods for measurement of multiple biomarkers for AD.

This approach has the potential for application to >3 biomarkers. Since AD has several biomarkers, it would be desirable to expand the number that can be tested simultaneously. The only limit on the number of additional biomarkers would be the availability of specific antibodies available for these various antigens. This positions the xMAP technology on the continuum between single-protein measurement (ELISA) and total protein/peptide measurement (MS/proteomics) tests. It may be possible to measure a pattern of altered expression in a limited set of proteins and to match that pattern with that of a neurodegenerative disease such as AD. A comparative study of xMAP and ELISA assays for detecting CSF biomarkers of AD has concluded that multiplex xMAP is an appropriate assay platform and provides results that can be correlated with research-based ELISA values, facilitating the incorporation of this diagnostic biomarker into routine clinical practice (Wang et al. 2012).

Targeting of Chemokine Receptor as Biomarker for Brain Imaging

Chemokines and chemokine receptors comprise a large number of molecules implicated in a wide range of physiological and pathological processes. Because of their induction or upregulation during CNS pathologies, members of the chemokine system have been considered as biomarkers. The chemokine receptor CCR1, by virtue of specific expression in Aβ plaques, may be a biomarker for AD pathology (Savarin-Vuaillat and Ransohoff 2007). Targeting of CCR1 with a radiolabeled, small-molecule CCR1-antagonist, BX-471 (Bayer AG), was shown to act as a specific brain-imaging biomarker of AD for both early detection and tracking of progression. It was tested phase I/II clinical trials but was not developed further. CCR1-antagonist ZK811460 was labeled with 18F to explore its possible use as specific diagnostic tool in AD. Tracer characterization comprising PET imaging of brain and metabolite analysis was performed in AD patients and controls. Neither qualitative evaluation nor quantitative compartment analysis of PET data showed any enhanced binding of the 18F-labeled CCR1-antagonist in the brain of AD patients or controls (Beuthien-Baumann et al. 2012).

Biomarkers of AD in CSF

CSF Sulfatide as a Biomarker for AD

Sulfatides are a group of glycosphingolipids that are highly expressed in brain and constitute 5% of myelin lipid. Sulfatide in oligodendrocytes is involved in development and membrane stabilization. The level of sulfatide in the brain and the CSF may be modulated by ApoE genotype. The lipid second messenger ceramide, a

degradation product of sulfatide, is elevated in AD brain and has been implicated in modulation of β-secretase and potentiation of Aβ production.

Due to their potential involvement in neurological diseases, development of accurate and sensitive methods for measurement of sulfatides are needed. A high-throughput, quantitative method has been described for the determination of sulfatides in CSF using a fully automated liquid/liquid extraction method and quantified using ultra-performance liquid chromatography coupled to tandem mass spectrometry (Blomqvist et al. 2017). This method was applied to a patient cohort of subjects with an AD biomarker profile. Although the total sulfatide levels were unaltered compared to an age matched control group, the authors showed that the ratio of hydroxylated/non-hydroxylated sulfatides was increased in the patient cohort indicationg its usefulness as a biomarker of AD.

CSF Reelin as Biomarker of AD

Reelin is a glycoprotein expressed by RELN gene that is essential for the correct cytoarchitectonic organization of the developing CNS. Its function in the adult brain is less understood, although it has been proposed that Reelin is involved in signaling pathways linked to neurodegeneration. Reelin expression was analyzed in brains and CSF from AD patients and nondemented controls and a 40% increase in the Reelin protein levels was found in the cortex of AD patients compared with controls (Botella-Lopez et al. 2006). Similar increases were detected at the Reelin mRNA transcriptional level. This expression correlates with parallel increases in CSF but not in plasma samples. Reelin increases in other neurodegenerative disorders and correlates positively with Tau protein in CSF in AD.

Depletion of RELN, but not its downstream signaling molecules, is detectable long before the onset of Aβ pathology in preclinical AD in the human frontal cortex indicating a possible causative role of RELN decline in the precipitation of AD pathology and its potential as a preclinical biomarker for AD (Herring et al. 2012).

Monitoring of Synthesis and Clearance Rates of Aβ in the CSF

Whether dysregulation of Aβ synthesis or clearance causes sporadic AD is unclear. A safe and sensitive method to determine the production and clearance rates of proteins within the human CNS has been developed (Bateman et al. 2006). Ideally, the production and clearance rates stay balanced, causing the overall amount of Aβ in the CNS to remain constant. In the healthy volunteers the first measurements of the fractional production and clearance rates of Aβ in vivo in the human CNS was found to be 7.6% per hour and 8.3% per hour, respectively. In this technique, a form of the amino acid leucine that has been very slightly altered to label it is administered via an intravenous drip. Inside the leucine are carbon (C) atoms with 13 neutrons and protons (C13) in their nucleus instead of the more common 12 neutrons and protons (C12). Physiologically and biochemically, C13 acts just like C12, i.e. does not alter

the normal Aβ production and clearance processes and is very safe to use. Over the course of hours, brain cells pick up the labeled leucine and incorporate it into the new copies they make of Aβ and other proteins. Periodic samples of the subjects' CSF are taken through a catheter placed in the lumbar subarachnoid space, Aβ is purified from the samples and a MS is used to determine how much of the Aβ includes C13-labeled leucine. Tracking the rise of the percentage of Aβ with labeled leucine over time gives the subject's Aβ production rate. When the percentage of Aβ containing labeled leucine reaches a steady level, infusion of the labeled leucine is stopped. Periodic sampling of the patients' CSF continues, enabling a measurement of how quickly the CNS clears out the labeled Aβ. In the first test subjects, the test procedure lasted for 36 h. The technique is being applied to individuals with AD to search for novel biomarkers of disease, to assess underlying differences in protein metabolism that contribute to disease, and to evaluate treatments in terms of their pharmacodynamic effects on proposed disease-causing pathways. This new test could enable direct monitoring of patients in clinical trials to see if the drug is really doing what it is supposed to do in terms of Aβ metabolism. If further studies confirm the validity of this test, it could be very valuable for selecting drugs for further progression in clinical trials and at finding optimal doses.

Protein Biomarkers of AD in CSF

Proteomic approaches - microcapillary LC/MS of proteins labeled with ICAT - have been applied to quantify relative changes in the proteome of human CSF obtained from the lumbar cistern. Currently available technologies can detect APP and tau protein in the CSF. Quantitative proteomics of CSF from AD patients compared to age-matched controls, as well as from other neurodegenerative diseases, will enable the generation of a roster of proteins that may serve as specific biomarker panels for AD. The use of panels of molecular biomarkers derived from proteomic analysis may offer the best prospect for developing molecular diagnostic tests for complex neurodegenerative disorders such as AD. The following proteomic technologies are useful for discovery of polypeptides biomarkers in human CSF:

- Profiling: SELDI (Surface Enhanced Laser Desorption/Ionization) using a protein biochip.
- Identification: 1D and 2D SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-time of flight).
- Validation: Western blot and SELDI

Protein array technology has demonstrated the presence in the CSF of sensitive polypeptide biomarkers for AD. Some of these are as follows:

- Cyatatin C has been suggested as the most important extracellular inhibitor of cysteine proteases such as cathepsins. Alterations in the balance of cysteine proteases and cystatin C have been associated with several pathological conditions.

A mutant of human cystatin C causes hereditary cerebral hemorrhage with amyloidosis.

- β2 microglobulin is a polypeptide chain of 99 residues and is a normal constituent of plasma. It is also a major component of dialysis-related amyloid fibrils. Partially-folded β2 microglobulin is a key intermediate in the generation of the amyloid fibrils in vitro. β2 microglobulin were upregulated in AD
- VEGF is a growth factor that supports the growth and proliferation of vascular endothelial cells. Several fragments of the neurosecretory precursors of VEGF are found in human neurons. VEGF expression is mainly regulated by BDNF. Increased BDNF expression can be observed in dentate gyrus and CA3 regions, which are tissue that appear to die early in AD.

Combination of several methods has been used for AD biomarker discovery from blood, including novel sample preparation based on carrier-protein capture, biomarker enrichment, high-resolution MS, and powerful bioinformatic analysis in a unique cohort of well-characterized persons with and without AD. The mass fingerprint model successfully classifies blinded AD patient and control samples with high sensitivity and specificity. Potential applications of these biomarkers are diagnosis, assessment of severity and progression of the disease as well as a basis of new therapeutic approaches. If more than one of these biomarkers is present, the sensitivity and specificity of diagnosis of AD rises considerably.

A multiplex quantitative proteomics method, iTRAQ (isobaric Tagging for Relative and Absolute protein Quantification) has been used in conjunction with multidimensional chromatography, followed by tandem mass spectrometry (MS/MS), to simultaneously measure relative changes in the proteome of CSF obtained from patients with AD. A panel of 23 protein biomarkers in CSF has been described that acts as a neurochemical "fingerprint" to identify patients with AD (Finehout et al. 2007). Some of the biomarkers included proteins associated with the binding and transport of the Aβ in the senile plaques while others molecules were linked to inflammation and synaptic dysfunction that occurs as AD progresses. This study combined cutting edge proteomic technology, detailed image analysis, and complex computational and statistical analyses to simultaneously compare 2000 CSF proteins from cases with autopsy-proven AD to those from age-matched controls. This study is the first to use sophisticated proteomic methods to hone in on a group of CSF proteins that are specific to AD. Postmortem tests confirmed that the panel is >90% sensitive in identifying persons with AD. Monitoring changes in these CSF biomarkers may enable tracking the efficacy of experimental drugs.

In another using iTRAG proteomic methods, differential expression of zinc-alpha-2-glycoprotein, fibulin-1, platelet basic protein, thrombospondin-1, S100 calcium-binding protein A8, and S100 calcium-binding protein A9 were detected in the serums of AD patients in comparison with healthy controls (Shen et al. 2017). These proteins might play a role in AD pathophysiology and serve as potential biomarkers for AD diagnosis.

Tau Proteins in CSF

Tau proteins are microtubular binding proteins localized in the axonal compartment of neurons. Brain injury releases cleaved Tau proteins (C-tau) into the extracellular space where they are transported to the CSF. Tau protein in the CSF has been measured by a sandwich ELISA method. Increased CSF concentrations of phosphorylated tau (ptau) protein have been suggested as a biomarker for AD. Although elevated CSF tau levels are associated with AD pathology and can help discriminate AD from other dementing disorders, some patients with AD have a level less than the mean values of the cognitively normal cohort. Extensive and accurate analysis of CSF could be helpful to define tau proteins species present in physiological conditions, or released during the progression of a given neurodegenerative disease. Thus, determining the isoform content of tau should add specificity to the biological test, because many neurodegenerative disorders can be distinguished by their set of tau isoforms that accumulate in neurons.

Tests for the Detection of Aβ in CSF

Aβ peptide is a key molecule in the pathogenesis of AD. Reliable methods for detection and quantification of soluble forms of this peptide in human biological fluids and in model systems such as cell cultures and transgenic animals, are of great importance for further understanding the pathomechanism of AD. ELISA tests are available for quantification of Aβ40 and Aβ42 (Aβ peptides ending at residues 40 or 42, respectively) in human CSF. The antibodies specifically detect the expected peptides with equal affinity for soluble and fibrillar forms of the peptide. The presence of CSF obstructs the recognition of synthetic peptides by the antibodies and the immunoreactivity of endogenous CSF Aβ decreases with increasing storage time and temperature.

An ultrasensitive test based on nanotechnology, Luminex/Verigene, enables detection of as few as 50 molecules of Aβ in CSF. This is a biobarcode assay with a 500 zeptomolar target DNA sensitivity limit. Magnetic separation and subsequent release of barcode DNA from the gold nanoparticles leads to a number of barcode DNA strands for every target DNA. Chip-based barcode DNA detection can be done with PCR-like sensitivity but without the use of PCR.

Decreased CSF Aβ42 concentration, but not Aβ40 concentration is a biomarker for AD. This Aβ42 concentration decrease in CSF likely reflects precipitation of Aβ42 in amyloid plaques in brain parenchyma. This pathogenic plaque deposition begins years before the clinical expression of dementia in AD. Normal aging and the presence of the APOE4 allele are the most important known risk factors for AD. A study in aging human volunteers shows that CSF Aβ42 findings are consistent with acceleration by the APOE4 allele of pathogenic Aβ42 brain deposition starting in later middle age in persons with normal cognition (Peskind et al. 2006). High plasma concentrations of Aβ40, especially when combined with low concentrations of Aβ42, indicate an increased risk of dementia. A potential role of plasma

concentrations as a biomarker of incipient dementia warrants further investigation. The combination of CSF tau and Aβ provides a more accurate diagnostic tool.

In vivo labeling of APP with 13C leucine enables calculation of the fractional synthetic and clearance rates of Aβ in the CSF (Bateman et al. 2007). C2N Diagnostics uses carbon 13C, a naturally occurring non-radioactive carbon isotope, to label newly synthesized proteins and biomolecules for measuring production and clearance rates of biomolecules in humans. 13C is one Dalton heavier than regular 12C and this difference in weight can be resolved by tandem MS, enabling calculation of the ratio of labeled to unlabeled biomolecule. By measuring this ratio at different time points following infusion of a 13C labeled building block it is possible to measure the fractional synthetic rate of a given biomolecule. The fractional clearance rate can be calculated by stopping the 13C labeled building block infusion and measuring the decrease in the ratio of labeled to unlabeled biomolecule.

Tests Combining CSF tau and Aβ

A combination of plaque tau and Aβ may be a specific and sensitive CSF biomarker for AD. Several studies have found AD can be fairly accurately diagnosed through measurement of concentrations in CSF of Aβ and tau; the former is decreased and the latter is increased. It is estimated that up to 85% of suspected cases of AD can be definitively diagnosed by analysis of these proteins in CSF. A cross-sectional study was conducted to study to correlate antemortem levels of CSF Aβ42, total tau, and phosphorylated tau protein with AD-type neuropathologic changes in the brain (Tapiola et al. 2009). The combination of abnormally low CSF Aβ42 level and abnormally high CSF tau level predicted the presence of AD pathologic features with high accuracy. This combination assay may be helpful in diagnosing the presence of AD pathologic changes in the brain.

Scientists from the Alzheimer's Disease Neuroimaging Initiative (ADNI) have confirmed the role of tau and Aβ as a biomarker for onset of mild AD as well as established a method and standard of testing for these biomarkers. The CSF biomarker signature of AD defined by low $A\beta_{1-42}$ and high T-tau in the autopsy-confirmed AD cohort and confirmed in the cohort followed in ADNI for 12 months detected mild AD in a large, multisite, prospective clinical investigation, and this signature appears to predict conversion from MCI to AD (Shaw et al. 2009). Additionally, the researchers factored in known genetic risk factor APOE-ε4 into their analysis. The gene occurs in about 40% of all people who develop AD at age 65 or later. ADNI volunteers with APOE-ε4, high levels of tau, and low levels of amyloid were most likely to have mild AD. Conversely, three ADNI volunteers with MCI at the start of the study, but whose CSF biomarker levels were similar to volunteers free of the disease, reverted back to normal cognition by the end of the study. In a multicenter study, CSF $A\beta_{42}$, T-tau, and P-tau identified incipient AD with good accuracy, but less accurately than reported from single-center studies indicating a need for standardization of analytical techniques to overcome intersite assay variability (Mattsson et al. 2009). There are still some controversies about tau

and Aβ in CSF of patients with AD. According to one hypothesis, CSF ApoE4 increases risk of clinical progression through its association with CSF Tau in APOEε4 carriers, whereas development of AD in APOEε4 noncarriers may be unrelated to ApoE concentration (van Harten et al. 2017).

Blood Biomarkers of AD

A Serum Protein-Based Algorithm for the Detection of AD

A method has been reported for the identification of protein biomarkers in the blood that can be used to distinguish between individuals with and without AD (O'Bryant et al. 2010). The researchers compared protein patterns in blood samples from hundreds of individuals with or without AD and incorporated these potential biomarkers into an algorithm for detecting AD cases in a test group. Their initial data suggest that serum protein-based biomarkers can be combined with clinical information to accurately classify AD. A disproportionate number of inflammatory and vascular biomarkers were weighted most heavily in the analyses. Additionally, these biomarkers consistently distinguished cases from controls in significant analysis of microarray, logistic regression, and Wilcoxon analyses, suggesting the existence of an inflammatory-related endophenotype of AD that may provide targeted therapeutic opportunities for this subset of patients. Adding information on APOE ε4 status or demographic factors such as sex, age, or education further improved both the sensitivity and specificity of the algorithm, raising the former up to 94% and the latter to 84%. There is also hope to test the utility of the biomarker algorithm for distinguishing between different types of dementia and predicting AD progression.

Amyloid Precursor Protein

Amyloid precursor protein (APP) is usually not considered to be a biomarker of AD. The ratio of the two forms of APP in platelets may aid in the differentiation of AD from other dementias. The APP ratio (APPr) - the ratio of the optical density of the upper and lower APP immunoreactive bands - does not differ by age or gender and levels were similar between individuals without dementia and individuals with non-AD dementia. In contrast, mean APPr is significantly lower among patients with AD, compared with all other groups combined and with non-demented and non-AD dementia groups individually. Moreover, mean APPr is significantly different between mild, moderate, and severe AD patients, declining with increasing severity of the dementia. Using the proposed "best" APPr cut-off score of 0.57, the test results in an overall sensitivity of 88.2% with a specificity of 89.4%. The positive predictive value of the test would range from 0.40 at an AD prevalence of 10% to 0.84 at a prevalence of 50%. This peripheral biomarker might be useful as an

adjunctive test in the early phase of the disease for differentiation from normal aging. The clinical usefulness lies in AD presentation with atypical features, such as behavioral problems, depression, anxiety, or pyramidal/extrapyramidal disorders.

A clinical study provides further evidence in support of soluble APP (sAPPβ) as a promising new biomarker of AD, which may improve the diagnostic accuracy of existing biomarkers and also enable a less invasive diagnostic workup (Perneczky et al. 2013).

Detection of Aggregated Misfolded Proteins in the Blood

Aggregated misfolded proteins (AMPs), resulting from abnormal synthesis or metabolism of the normal neural protein, are associated with AD but are difficult to identify with traditional methods. A fundamental problem in the detection of AMPs in peripheral blood is the presence of the normal protein at a million-fold relative concentration. Amorfix Life Sciences Ltd. is developing an AD diagnostic that is based on its Epitope Protection technology. The company has acquired all rights to a process using chemical modifying agents that alter epitopes on normal proteins but not on AMPs. This procedure minimizes the background and once the aggregate has been disaggregated, the conserved epitopes can then be detected by ultra-sensitive immunodetection procedures using standard reagents. This process can be applied to any AMP and overcomes the fundamental problem of having to detect AMPs in a solution of proteins where normal proteins are much more prevalent.

Lipid Biomarkers for Preclinical Detection of AD

A lipidomic approach has been described to detect preclinical AD in a group of cognitively normal older adults. The authors discovered and validated a set of 10 lipids from peripheral blood that predicted phenoconversion to either amnestic MCI or AD within a 2–3 year timeframe with >90% accuracy (Mapstone et al. 2014). This biomarker panel, reflecting cell membrane integrity, may be sensitive to early neurodegeneration of preclinical AD.

Lymphocyte Proliferation Test

The Lymphocyte Proliferation (LymPro™, Provista Life Sciences) diagnoses AD by measuring AD-related disturbances of neuronal cell cycles. Through quantifying and evaluating CD69 presentations on the surface of peripheral blood lymphocytes (PBLs) with and without mitogenic stimulation. In this test, PBLs, which are easily obtained from patients' blood samples, are used as proxy for AD susceptible neurons because they express a variety of cell surface receptors comparable to neurons. Control of proliferation and differentiation of lymphocytes moreover, involves signal transduction systems similar to neurons. Lymphocytes of AD

patients are impaired in their proliferative response to APP. In a clinical study involved patients with probable AD the stimulation index for all mitogenic compounds was found to be significantly reduced in AD patients compared to controls. There was no detectable stimulation in the advanced cases of AD. Initially tested on isolated lymphocytes, the protocol has also been successfully employed using whole blood samples.

Metabolomic Biomarker Profiling

VTT Technical Research Centre (Finland) has Serum metabolomic profiles associated with progression of MCI to AD examined in a prospective study using a comprehensive metabolomics approach to analyze baseline serum samples and to associate the metabolite profiles with the diagnosis at baseline and in the follow-up (Orešič et al. 2011). At baseline, AD patients were characterized by diminished ether phospholipids, phosphatidylcholines, sphingomyelins and sterols. A molecular signature comprising three metabolites was identified, which was predictive of progression to AD in the follow-up. The major contributor to the predictive model was 2,4-dihydroxybutanoic acid, which was upregulated in AD progressors, indicating potential involvement of hypoxia in the early AD pathogenesis. This was supported by the pathway analysis. VTT is now collaborating with GE Healthcare to validate a serum biochemical signature that is believed to predict progression to AD months or even years before the disorder's first symptoms occur, in a large patient cohort, as well as to discover additional biomarker candidates. VTT and GE hope to develop the biomarker into a clinical assay. VTT's work comes as part of a "biosignatures initiative" alliance of GE Healthcare with Janssen Pharmaceutica to develop diagnostic biosignatures for presymptomatic identification of AD.

Plasma Protein Biomarkers of AD

Blood specimens are used for genotyping in AD but no single blood test has proven to be an acceptable biomarker for AD. This may be because such tests are far removed from central events in the disease. Blood Aβ levels in humans also do not reflect the amount of amyloid plaques in the brain. Some of the tests may assess physiological processes involved in neurodegeneration in general and thus may be better markers of the severity of neurodegeneration rather than markers for a specific cause of neurodegeneration. Plasma Aβ measures increase with age, but, in contrast to reports on familial AD, plasma Aβ measures are neither sensitive nor specific for the clinical diagnosis of mild cognitive impairment or sporadic AD. Although the significance of Aß for diagnosing AD is controversial, high plasma concentrations of Aß40 and low plasma concentrations of Aß42 indicate an increased risk of dementia. Role of plasma Aß as a biomarker of incipient dementia warrants further investigation.

Health ABC Study, a prospective observational study to determine if plasma Aβ is associated with cognitive decline and if this association is modified by measures

of cognitive reserve was begun in 1997–1998 with 10-year follow-up in 2006–2007 (Yaffe et al. 2011). Main outcome measures were association of near-baseline plasma Aβ levels (42 and 42/40 measured in 2010) and repeatedly measured Modified Mini-Mental State. The results suggest that older adults without dementia and with lower Aβ42/40 levels have an increased rate of cognitive decline over 9 years compared with those with higher levels and that this relationship is modified by measures of cognitive reserve. Specifically, the association between Aβ plasma level is greater among those with less education, lower literacy, and an APOE e4 allele. In addition, these finding of an interaction of cognitive reserve with the association of plasma Aβ level and cognitive decline could have public health importance because it may suggest pathways for modifying Aβ effects on cognition.

Proteins pathogenic in AD have been extracted from neutrally-derived blood exosomes and quantified by ELISA to develop biomarkers for the staging of sporadic AD (Fiandaca et al. 2015). Results showed that levels of P-S396-tau, P-T181-tau, and Aβ1–42 in extracts of neurally derived blood exosomes predict the development of AD up to 10 years before clinical onset. Commercial development of this test is being conducted by NanoSomix Inc.

Plasma samples from early Alzheimer disease patients have been examined with proteomic technologies – 2D-DIGE and MALDI-TOF/TOF/MS – and compared with samples from normal subjects as controls. Two proteins, including apolipoprotein A-4 and fibrinogen gamma chain, were upregulated in plasma of mild AD patients suggesting that altered expression levels of these proteins may yield candidate biomarkers for the early diagnosis of the disease (Kitamura et al. 2017).

Protein Kinase C in Red Blood Cells

Protein kinase C (PKC) plays an important role in the pathophysiology of AD, as the alteration of its activity stimulates Aβ production and tau hyperphosphorylation. Therefore, PKC is considered a potential therapeutic target for disease modifying drugs. PKC alterations have been observed in peripheral cells including RBCs. Using fluorescent probes specific for PKC, it is possible to detect the conformational changes of the enzyme in living cells such as RBCs (de Barry et al. 2010). This alteration is independent of the patient's age and of the stage of the disease. PKC alteration can be used as a biomarker to distinguish AD patients from healthy controls with unmatched specificity and sensitivity.

Urine Tests for AD

Neural thread protein (NTP), a membrane-associated phosphoprotein, is selectively elevated in urine of AD patients. AD7C-NTP is associated with the pathologic changes of AD, and overexpression of the AD7C-NTP gene is associated with cell death similar to that found in the AD brain. A prospective blinded multicentered study has shown that urine NTP could be used as a safe and promising biochemical

marker of early AD (Youn et al. 2011). A competitive ELISA (Nymox's AlzheimAlert™) for determining AD7C-NTP levels in urine is commercially available for the diagnosis of AD. AlzheimAlert™ test kit is now certified with a CE Mark, making the device eligible for sale in the European Union. The CE Marking indicates that a product complies with EU safety, environmental, and quality standards. The European clinical and hospital laboratories are allowed to perform the AlzheimAlert test in their own facilities in Europe. This test may become an important tool for monitoring emerging therapies for AD.

A Biomarker-Based Skin Test for AD

A biomarker isolated from skin fibroblasts can accurately distinguish between AD and other forms of dementia during the first one to two years of the disease's progression. The inflammatory agonist bradykinin, a small nanopeptide that induces PKC-mediated phosphorylation of Erk1 and Erk2 in fibroblasts, was applied to punch-biopsy-obtained human skin fibroblasts (Khan and Alkon 2010). Quantitative imaging of the phosphorylated Erk1 and Erk2 bands was then used in a ratio that is mathematically configured into an AD-Biomarker Index (AD-Index). Demented individuals were clinically diagnosed as AD with an overall accuracy of 78%. Among the autopsy-confirmed cases for which there were also AD-Biomarker measurements, the overall accuracy of the AD-Biomarker was 98%. Application of soluble $A\beta_{1-42}$ to the fibroblasts of normal controls induced the abnormal AD-Biomarker phenotype, suggests the pathophysiologic relevance of this AD-Biomarker measurement. The AD-Biomarker, as confirmed by autopsy validation, showed significantly higher sensitivity and specificity than did clinical diagnosis, particularly at early stages of disease, and pathophysiological relevance was demonstrated for the mechanistic basis of the AD-Biomarker measurements. A test based on this biomarker could be performed easily by a nurse or medical technician in a physician's office or outpatient clinic. The molecular pathway measured by this biomarker includes the same enzyme, PKC, which is targeted by the drug bryostatin. This drug is in phase II clinical trials of to determine if it is useful in treating both the symptoms and neurodegeneration of AD.

Salivary Biomarkers of AD

Saliva Aβ42 level could be considered a potential peripheral biomarker of AD and help discrimination from other types of neurodegenerative disorders. In a pilot study, saliva and plasma levels analyzed of Aβ were measured using a highly sensitive ELISA kit (Bermejo-Pareja et al. 2010). There was a statistically significant increase in saliva Aβ42 levels in mild AD patients, but there were no differences in

saliva concentration of Aβ42 between patients with Parkinson disease and healthy controls. Saliva Aβ40 expression was unchanged within the studied sample. The association between saliva Aβ42 levels and AD was independent of established risk factors, including age or Apo E.

Oasis Diagnostics has licensed a technology for the development of saliva-based POC diagnostics for AD using 8 proprietary biomarkers found in saliva for the diagnosis, prognosis, and monitoring of the effectiveness of new therapies for patients with AD. The company will develop the tests on the company's Verofy POC platform, which uses immunochromatographic test strips.

Applications of Biomarkers of AD

Biomarker Changes in Autosomal Dominantly Inherited AD

Because autosomal dominantly inherited AD develops over many years, the age of onset is predictable and provides an opportunity to determine the sequence and magnitude of pathologic changes that lead to symptomatic disease. Analysis of data from a prospective, longitudinal study has revealed the following (Bateman et al. 2012):

- Concentrations of Aβ42 in the CSF appeared to decline 25 years before expected symptom onset.
- Aβ deposition, as measured by PET with the use of Pittsburgh compound B, was detected 15 years before expected symptom onset.
- Increased concentrations of tau protein in the CSF and an increase in brain atrophy were detected 15 years before expected symptom onset.
- Cerebral hypometabolism and impaired episodic memory were observed 10 years before expected symptom onset.
- Global cognitive impairment, as measured by the MMSE and the Clinical Dementia Rating scale, was detected 5 years before expected symptom onset.
- Patients met diagnostic criteria for dementia at an average of 3 years after expected symptom onset.

These results require confirmation with the use of longitudinal data and may not apply to patients with sporadic AD.

The findings of a study of young adults from a population in Colombia with a high prevalence of a mutation in the PSEN1 gene, which leads to the development of AD at an early age, suggests that biomarkers of AD might be apparent even earlier than thought, perhaps 20 years before the onset of symptoms (Jagust 2012). There was an increase in CSF levels of Aβ in mutation carriers well in advance of clinical onset of the disease. It is hoped that early detection biomarkers could enable identification of presymptomatic AD patients and improve their therapeutic options.

Correlation of Imaging Biomarkers with CSF Biomarkers of AD

A study has investigated correlation of structural changes in brain determined by brain imaging with CSF biomarkers in patients suffering from subjective cognitive impairment, MCI, and AD (Li et al. 2014). Using diffusion tensor imaging, correlations were obtained between CSF biomarkers, including Aβ42, tau phosphorylated at position threonine 181 as well as total tau protein, and grey matter volume. Self-diffusion fractional anisotropy (FA) and mean diffusivity (MD) maps were drawn using voxel-based morphometry and tract-based spatial statistical analyses. In the whole sample, there was positive correlation between grey matter volume and Aβ42 concentration, and negative correlation with total tau protein. The study concluded that early pathological changes in AD can be detected with voxel-based morphometric analysis and diffusion tensor imaging measurements. Furthermore, there was an association between CSF AD biomarkers and structural brain changes in areas related to the default mode network.

Genetic Tests for AD

Genetic tests are available to identify individuals with familial forms of AD who have AD-linked mutations in the presenilin gene, and those who have specific variations in the ApoE gene linked to higher risk of developing AD. Such diagnostics do not measure the deadly amyloid fibrils themselves - the true test for AD, which now can only be done post-mortem.

ApoE genotype tests are based on the reverse hybridization principle. Amplified biotinylated DNA material is hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane-based strips. After hybridization, streptavidin labeled with alkaline phosphatase is added and bound to the previously formed biotinylated hybrid. Incubation with a chromogen produces a purple precipitate. Alleles ε2, ε3, and ε4 can be detected by Pronto™ ApoE test (Savyon Diagnostics) with demonstration of the six possible genotypes. The ApoEε4 allele, a risk factor rather than a disease gene, has a positive predictive value of 94% to 98% in an individual with suspicion of AD. It is useful for predicting response to certain drugs for AD. Although no consensus has been reached as to the value of genetic testing for early detection of late-onset AD, ApoE testing might become important in the future if it helps to define the need for intervention or to select an optimal intervention.

Humanin as a Biomarker as Well as Neuroprotective in AD

Humanin (HN), a 24-residue peptide, is a neuroprotective factor and a potential biomarker of anti-cell death activity against a wide spectrum of AD-related cytotoxicity, including exposure to Aβ in vitro. Injection of S14G-HN, a highly potent HN derivative, into brain has been shown to ameliorate memory loss in an Aβ-injection mouse

model. To fully understand HN's functions under AD-associated pathological conditions, a study has examined the effect of S14G-HN on triple transgenic mice harboring APP(swe), tau(P310L), and PS-1(M146 V) that show the age-dependent development of multiple pathologies relating to AD (Niikura et al. 2011). Behavioral analyses showed that intranasal treatment with S14G-HN ameliorated cognitive impairment in male mice. Moreover, ELISA and immunohistochemical analyses showed that Aβ levels in brains were markedly lower in S14G-HN-treated male and female mice than in vehicle control mice. The authors also found the expression level of neprilysin (NEP), an Aβ degrading enzyme, in the outer molecular layer of hippocampal formation was increased in S14G-HN-treated mouse brains. NEP activity was also elevated by S14G-HN treatment in vitro. These findings suggest that decreased Aβ level in these mice is at least partly attributed to S14G-HN-induced increase of NEP level. Although HN was identified as an anti-neuronal death factor, these results indicate that HN may also have a neuroprotective effect.

Plasma Biomarkers of Drug Response in AD

Plasma Aβ may be of limited diagnostic value, but it may provide important information as a measure of treatment response. Several measures of detectable products of cellular processes are being developed as possible biomarkers of therapeutic response accessible in the plasma (Cummings 2011). Surrogate biomarkers that can function as outcomes in pivotal trials and reliably predict clinical outcomes are needed to facilitate primary prevention trials of asymptomatic persons where clinical measures may be of limited value. Identification and assessment of a number of candidate biomarkers in patients with AD has been described as well as the correlation of those biomarkers with rosiglitazone therapeutic efficacy, as represented by a change in the Alzheimer's Disease Assessment Scale-Cognitive (ADAS-Cog) score (Akuffo et al. 2008). Plasma from patients with AD was analysed by proteomics at baseline and after receiving rosiglitazone for 24 weeks. From a comparison of protein expression following treatment with rosiglitazone, 97 proteins were observed to be differentially expressed, from which a prioritized list of 10 proteins were analysed by immunoassay and/or functional assay in a wider set of samples from the same clinical study, representing a rosiglitazone dose response, in order to verify the changes observed. A number of these proteins appeared to show a correlation with change in ADAS-Cog at the higher treatment doses compared with the placebo. Alpha-2-macroglobulin, complement C1 inhibitor, complement factor H and apolipoprotein E expression showed a correlation with ADAS-Cog score at the higher doses.

PredictAD Project

PredictAD (https://www.predictad.eu/) is an EU funded project with participation of research institutions and companies, which aims to develop an objective indicator to diagnose AD at the earliest stage. The project started with focus on discovering

biomarkers from biomolecular data, electrophysiological measurements of the brain and structural, functional and molecular brain images. Later a statistical model designed and built as a framework for exploiting these biomarkers with other available patient history and background data. The participants were able to discover several potential novel biomarker candidates and implement the framework in software (Antila et al. 2013). The results are currently used in several research projects, licensed to commercial use and being tested for clinical use in several trials.

TOMM40 Gene and Risk of AD

A case-control analysis, using hippocampal atrophy as a quantitative phenotype in a genome-wide scan, has identified a new risk gene, TOMM40 (translocase of outer mitochondrial membrane 40), which is approximately twice as frequent in AD subjects as controls (Potkin et al. 2009). The TOMM40 gene influences the ease with which molecules can get in and out of mitochondria, the energy production center and stress mediator of cells. TOMM40 also processes materials that form amyloid plaque. Having the harmful form of TOMM40 significantly increases one's susceptibility when other risk factors such as ApoE4 are present. With aging, the number and function of mitochondria decrease, accompanied by a parallel increased risk of developing AD. This study points to the use of mitochondrial-based therapies for treating the disease. The OPAL (Opportunity to Prevent Alzheimer's disease of Late onset), designed to see if drug intervention can help in those with TOMM40 gene.

Commerical development of TOMM40 assay as a biomarker for AD is being done by Zinfandel Pharmaceuticals Inc. in collaboration with Takeda Pharmaceuticals. In February 2016, they completed of enrollment in TOMMORROW trial, the largest phase III trial of its kind. It is investigating a genetic-based biomarker risk assignment algorithm (BRAA) and evaluating the safety and efficacy of investigational drug pioglitazone to delay the onset of MCI due to AD in cognitively normal individuals projected to be at high risk, as determined by the BRAA. The BRAA has three components: APOE, TOMM40 genotypes and age. Increased age and certain APOE genotypes have previously been shown to indicate elevated risk of AD, but neither is sufficiently sensitive nor specific. The addition of TOMM40 genotype is hypothesized to further refine the risk determination. Studies show that individuals with MCI are at an increased risk of developing AD or another dementia, with conversion rates of ~15% per year. No medication studied to date has been shown to reliably delay the onset of AD.

Use of Biomarkers to Predict AD in Patients with MCI

Disease-modifying treatment strategies for AD have led to an urgent need for biomarkers to identify the disease at a very early stage. Efforts are being made to assess the association between CSF biomarkers and incipient AD in patients with mild

cognitive impairment (MCI). CSF concentrations of Aβ1–42 (Aβ42), total tau (T-tau), and phosphorylated tau (P-tau) were analyzed in MCI patients using Luminex™ xMAP technology (Hansson et al. 2006). During follow-up, 42% patients with MCI developed AD, 15% developed other forms of dementia, and 41% remained cognitively stable for 5•2 years. A combination of CSF T-tau and Aβ42 at baseline yielded a sensitivity of 95% and a specificity of 83% for detection of incipient AD in patients with MCI. The relative risk of progression to Alzheimer's disease was substantially increased in patients with MCI who had pathological concentrations of T-tau and Aβ42 at baseline. The association between pathological CSF and progression to AD was much stronger than, and independent of, established risk factors including age, sex, education, APOE genotype, and plasma homocysteine. The combination of T-tau and Aβ42/P-tau181 ratio yielded closely similar results (sensitivity 95%, specificity 87%). The conclusion is that concentrations of T-tau, P-tau181, and Aβ42 in CSF are strongly associated with future development of AD in patients with MCI.

In a 2-year longitudinal study relative to control, MCI patients showed decreased memory and hippocampal volumes and elevated CSF levels of hyperphosphorylated tau and isoprostane (de Leon et al. 2006). These two CSF measures consistently improved the diagnostic accuracy over the memory measures and the isoprostane measure incremented the accuracy of the hippocampal volume achieving overall diagnostic accuracies of about 90%. Among MCI patients, over 2 years, longitudinal hippocampal volume losses were closely associated with increasing hyperphosphorylated tau and decreasing Aβ42 levels. These results demonstrate that CSF biomarkers for AD contribute to the characterization of MCI.

Concluding Remarks about Biomarkers for AD and Future Prospects

The ideal biomarker for AD has not been discovered as yet. Relationship between peripheral biomarkers and the pathological process in AD can be depicted as 3 options:

1. Biomarkers are brain-specific and brain tissue damage may lead to altered concentrations in body fluids.
2. Biomarker concentrations are changed due to peripheral imbalances, and these imbalances might influence the neurodegenerative process as a secondary effect.
3. Biomarkers are related to disturbed processes in the brain as well as in the periphery.

The first options will yield the most specific indicators for brain damage whereas the other two options may be equally relevant for the pathological process. These concepts and further studies on the relation between biomarker concentrations and

characteristic tissue damage for AD can increase the opportunity to find new biomarkers useful for diagnosis and therapeutic follow-up.

It is highly unlikely that only a few biomarkers will be sufficient for an accurate test for AD. Rather, panels of 50 or more proteins and phosphoproteins will need to be tracked in parallel in blood or CSF samples. Furthermore, MRI- or PET-based markers of AD may be necessary to increase the accuracy of in vivo diagnosis. Broad screening with panels of hundreds of antibodies at a time for established biomarkers in blood-based tests will become standard for presymptomatic detection of AD in the near future.

Demonstration that T-tau, P-tau$_{181}$, and Aβ42 in CSF are strongly associated with future development of AD in patients with MCI may have an effect both on the diagnostic work-up and on the design of clinical trials of patients with MCI. Furthermore, some studies indicate that CSF biomarkers could identify AD preclinically, even before the onset of MCI. The same might hold true for PET and functional MRI. In clinical drug trials with follow-up periods of 3–4 years, around 50–80% of patients with MCI do not develop AD. Inclusion of large numbers of patients with MCI who do not have incipient AD in clinical trials of the some disease-modifying agents, such as Aβ42 immunotherapy, which might involve a risk of serious side-effects could be regarded as unethical. Moreover, the beneficial effects of a treatment might be more difficult to assess in such heterogeneous populations of MCI patients. In the future, CSF, MRI, and PET biomarkers could be valuable tools to enrich clinical MCI trials with patients at high risk of developing AD. The North American ADNI, and similar activities in Europe, Japan, and Australia, are natural history studies that are providing new insights into the use of imaging end points in clinical trials. Although results of these trials are not yet all available, evidence of success of imaging to assess eligibility, safety and efficacy is emerging.

Although biomarkers can be useful for confirming or excluding a presumptive diagnosis based on clinical testing or history of AD, there are no standardized procedures available as yet to run these tests. The aim of BIOMARKAPD program, launched in 2012 as part of the European Union's "Joint Programme in Neurodegenerative Disease Research initiative", is to refine data on known biomarkers typically found in CSF of AD patients. The Karolinska Institute (Stockholm, Sweden), is addressing some of the challenges associated with standardizing how biomarkers are measured and reported at different laboratories. As part of the program, researchers at McGill University (Montreal, Canada) are developing standardized protocols for patient samples. If a laboratory reports that an individual has CSF concentration of X for β42 and Y for Tau phosphorylation, the standard procedure should enable reproduction of the results at another laboratory. This should eliminate variation due to different methods across laboratories, which is obviously a major stumbling block to advance molecular diagnostics for AD. This is particularly important for the use of biomarkers for detection or measurement of the disease process in its presymptomatic stages of AD prior to onset of dementia.

Biomarkers of Parkinson's Disease

Parkinson disease (PD) is a chronic, progressive neurodegenerative disorder that affects at least 1% of people by age 70. Classical clinical features of PD include resting tremor, rigidity, balance impairment, and slowness of movement. Clinical diagnosis is based mostly on motor symptoms such as tremor, rigidity, bradykinesia, and postural instability as well as their response to dopamine replacement therapy. By the time clinical diagnosis is made >50% of dopaminergic neurons in the substantia nigra have already degenerated and other neurotransmitter systems (e.g., norepinephrine, serotonin and acetylcholine) are involved, indicating that the disease is already at an advanced stage. Clinical diagnosis of PD at early stages is inaccurate and nearly half of the patients are misdiagnosed. This is one of reasons for failure of clinical trials of neuroprotective agents for PD. There is still no objective endpoint for clinical trials in early PD other than the Unified PD Rating Scale (UPDRS), a rater-based assessment of symptom severity. Thus, there is a need for objective biomarkers of PD that either are neuropathophysiological manifestations of the disease, track the speed of its progression, or can identify those at-risk of PD (Mollenhauer et al. 2010). Because of the complexity and variability of the disease, it is likely that a panel of biomarkers all PD subtypes. Because the disease involves the peripheral as well as the central nervous system, additional biomarkers are needed that can be accessed via blood or peripheral tissue analysis. Table 14.5 shows biomarkers of PD.

Eosinophilic inclusions (Lewy bodies) were identified in the brains of PD patients and, along with abnormalities in the substantia nigra, became a recognized pathologic biomarker of the disease.

Autoantibodies as Biomarkers of PD

Human protein microarrays were used to reveal serum autoantibodies, which are differentially expressed among PD and control subjects, and the diagnostic significance of each of these autoantibodies was evaluated, resulting in the selection of 10 autoantibody biomarkers that can effectively differentiate PD sera from control sera with a sensitivity of 93.1% and specificity of 100% (Han et al. 2012). PD sera were also distinguishable from sera obtained from AD, breast cancer, and multiple sclerosis patients with accuracies of 86.0%, 96.6%, and 100%, respectively. These results demonstrate that serum autoantibodies can be used as highly specific and accurate biomarkers for PD diagnosis throughout the course of the disease. Results of another study demonstrate that a panel of selected, blood-borne autoantibody biomarkers can distinguish early stage PD subjects from controls with an overall accuracy of 87.9%, a sensitivity of 94.1% and specificity of 85.5% (DeMarshall et al. 2015). These biomarkers were also capable of differentiating patients with

Table 14.5 Biomarkers of Parkinson disease

Serum autoantibodies
Biochemical biomarkers
5-hydroxy-indole acetic acid
8-hydroxy-2′ deoxyguanosine increased in CSF
Mitochondrial complex I measurement
Methoxy-hydroxy-phenylethylene glycol
Vitamin D (25-hydroxyvitamin D) decreased in serum
Cardiac denervation as a biomarker of PD: shown with MIBG SPECT
Molecular biomarkers based on gene expression in blood
Imaging biomarkers
AADC (aromatic L-amino acid decarboxylase) reduction in striatum
Aβ identified by PET
Altered dopamine receptor binding on 11C-raclopride PET on 18F-dopa PET
Dopamine transporter ligand uptake reduction on 123I β-CIT SPECT
Glucose metabolism reduction in striatum on 18F-deoxyglucose PET
Iron deposits in the midbrain of early PD shown by high-field MRI technology
L-dopa uptake and decarboxylation reduction on 18F-dopa PET
Mineral deposition in the SN on transcranial ultrasound
Peripheral benzodiazepine receptors/activated microglia on PK11195 PET, indicating neuroinflammation
VMAT2 (vesicular monoamine transporter 2) reduction on PET
Protein biomarkers: e.g., hypocretin
Screening for genetic mutations
α-synuclein gene mutations
DJ-1 oncogene for early onset PD
LRRK2 (leucine-rich repeat kinase 2) for late onset PD
NR4A2 gene mutations
Parkin
PINK-1 (PTEN Induced Putative Kinase 1)
ST13 RNA: gene expression is lower in blood of PD patients
UCH-L1 (ubiquitincarboxy-terminalhydrolase L1) gene mutations

early stage PD from those with more advanced (mild-moderate) PD with an overall accuracy of 97.5%, and could distinguish subjects with early stage PD from those with other neurological and non-neurological diseases. Serum autoantibodies are the most promising biomarkers for diagnosis of PD.

Biomarkers of PD Based on Gene Expression in Blood

In PD patients several genes differentially expressed are involved in the pathobiologically and therapeutically relevant processes of the ubiquitin-proteasome pathway, mitochondrial function, and apoptosis. Insights into disease-linked processes detectable in peripheral blood are offered by 22 unique genes differentially expressed in patients with PD versus healthy individuals (Scherzer et al. 2007). These include the cochaperone ST13, which stabilizes HSP70, a modifier of α-synuclein misfolding and toxicity. ST13 mRNA copies are lower in patients with PD than in normal subjects. Thus, gene expression signals measured in blood can facilitate discovery of biomarkers for PD.

Cardiac Denervation as a Biomarker of PD

Cardiac sympathetic denervation is very common in patients with PD and neurogenic orthostatic hypotension. Approximately 50% of patients with PD who do not have orthostatic hypotension also have evidence for loss of noradrenergic innervation. The loss progresses over years. Reduced cardiac innervation can be shown with ^{123}I–metaiodobenzylguanidine (MIBG) SPECT. Because patients with familial PD who have mutations of the gene encoding α-synuclein or triplication of the normal gene have low myocardial concentrations of 6-^{18F}fluorodopamine-derived radioactivity, cardiac sympathetic denervation is linked with α-synucleinopathy. Baroreflex-cardiovagal failure and cardiac sympathetic denervation can occur before onset of the movement disorder, suggesting that neurocardiologic testing might provide a biomarker for detecting presymptomatic or early PD and for monitoring responses to neuroprotective therapy (Goldstein 2007).

Genetic Biomarkers of PD

Historically, the role of genetic heritability in PD has been considered negligible; however, within the last two decades a series of discoveries have dramatically changed this view. Mitochondrial dysfunction plays a central role in the pathobiology of PD. Identification of mutations in genes encoding PINK1 (PTEN-induced kinase 1) and Parkin in familial PD as well as their functional association with mitochondrial quality control supports this view. Several approaches for the development of biomarkers and disease-modifying therapies for PD are based on a detailed understanding of the PINK1/Parkin pathway (Truban et al. 2017).

In 2014, Lundbeck received support and recognition for its research on PD through a grant from The Michael J. Fox Foundation to explore a genetic cause in hereditary forms of the disease as a platform for developing disease-modifying

treatment that would slow or stop the progression of the disease in both hereditary and sporadic cases. Lundbeck is investigating variation in the gene Leucine Rich Repeat Kinase 2 (Lrrk2), the best known genetic contributor to PD. Part of that research also suggests that the Lrrk2 biology is central to both hereditary and sporadic PD. By studying the role of a mutation in Lrrk2, Lundbeck hopes to be able to identify biomarkers of the disease, paving the way for earlier diagnosis of patients who do not have hereditary form of the disease. That would speed up specific treatments targeting this gene, potentially paving the way for new and better treatments of PD. This research program will continue over the following 3 years. Lundbeck will conduct research to identify Lrrk2-dependent biological fingerprints in specific cells in the blood. The identified fingerprints will be used in several ways. First, they will provide more general information about the biological function of Lrrk2 and might help to understand important mechanisms underlying the general involvement of the Lrrk2 biology in disease onset and progression. Secondly, it is anticipated that besides the more immediate outcomes listed above, the project will, in the long run, provide valuable data on identifying markers to measure progression of PD. This would be useful for supporting clinical trials and developing disease-modifying treatments.

Imaging Biomarkers of PD

Radiotracer imaging of the nigrostriatal dopaminergic system is a widely used but controversial biomarker in PD. Four radiotracers are used as biomarkers in PD: ^{18F}fluorodopa PET, (+)-^{11C}dihydrotetrabenazine PET, ^{123I}beta-CIT SPECT, and ^{18F}fluorodeoxyglucose PET. They help to understand disease biology and facilitate drug discovery and early human trials relying on the evidence that they are measuring relevant biologic processes. The four tracers fulfill this criterion, although they do not measure the number or density of dopaminergic neurons. Biomarkers used as diagnostic tests, prognostic tools, or surrogate endpoints must not only have biologic relevance but also a strong linkage to the clinical outcome of interest. No radiotracers fulfill these criteria, and current evidence does not support the use of imaging as a diagnostic tool in clinical practice or as a surrogate endpoint in clinical trials. Mechanistic information added by radiotracer imaging to clinical trials may be difficult to interpret because of uncertainty about the interaction between the interventions and the tracer.

In two double-blind trials of glial cell derived neurotrophic factor (GDNF) infusion in PD, there was 30% improvement in F-dopa uptake in the putamen, specifically in the region immediately surrounding the catheter tip suggesting that some GDNF may have diffused into the surrounding putamen, resulting in improved dopaminergic function (Sherer et al. 2006). However, the increases in dopamine uptake did not correlate with clinical outcomes. For example, some patients who showed improvement in F-dopa uptake failed to show clinical improvement. One possibility is that increased F-dopa uptake is the result of specific biological action

of GDNF (suggesting that GDNF is reaching its biological targets), but that the accompanying effects on dopaminergic neurons are not sufficient to induce a clinical improvement in all patients. Another possibility is that F-dopa uptake is only measuring one index of neuronal function that does not correlate with clinical outcomes. As PD affects more than just the nigrostriatal system, this is perhaps not surprising. Finally, changes in F-dopa may represent an even more nonspecific alteration such as an inflammatory response to the surgery or infusion.

Olfactory dysfunction is common in PD and is more closely correlated with hippocampal dopaminergic than nigrostriatal dopaminergic denervation. Results of multi-tracer PET studies show that odor identification deficits in PD are best predicted by cholinergic denervation and to a lesser extent by dopaminergic denervation. Progressive changes in olfaction may be used as a biomarker of cholinergic denervation and cognitive decline in PD (Bohnen and Müller 2013).

Metabolic Brain Networks as Biomarkers

Metabolomic profiling of plasma yielded strong prediction of PD progression and offers biomarkers that may provide new insights into PD pathogenesis (LeWitt et al. 2017). PD is characterized by elevated expression of an abnormal metabolic brain network that is reduced by clinically effective treatment. FDG PET has been used to determine the basis for motor improvement in PD patients receiving unilateral subthalamic nucleus (STN) infusion of an adeno-associated virus vector expressing glutamic acid decarboxylase (AAV-GAD). Gene therapy significantly reduces thalamic metabolism on the operated side but increases metabolism in motor and premotor cortical regions of the involved side of the brain. Abnormal elevations in the activity of metabolic networks associated with motor and cognitive functioning in PD patients are evident at baseline. The activity of the motor-related network declined after surgery and persist at 1 year. These network changes correlate with improved clinical disability ratings, but the activity of the cognition-related network does not change after gene transfer suggesting that modulation of abnormal network activity underlies the clinical outcome observed after unilateral STN AAV-GAD gene therapy. Network biomarkers may be used as physiological assays in early-phase trials of experimental therapies for PD.

Metabonomic Biomarker Profile for Diagnosis and Monitoring of PD

The development of biomarkers for the diagnosis and monitoring disease progression in PD is importance because diagnosis based on clinical features has a high rate of errors. At best, a symptom-based screen is only 90% accurate. A study to search for biomarkers of PD based on metabolomic profiling used high performance liquid

chromatography coupled with electrochemical coulometric array detection (LCECA) to detect biomarkers in plasma that are useful for the diagnosis of PD (Bogdanov et al. 2008). The investigators measured 8-hydroxy-2-deoxyguanosine (8-OHdG) levels as a biomarker of oxidative damage to DNA. They initially examined the profiles of PD subjects not exposed to drugs and compared them to controls to rule out confounding effects of drugs. The two groups had distinctly different metabolomic profiles. No one molecule was definitive, but a pattern of about 160 compounds emerged that was highly specific to PD patients. They then determined the variables, which played the greatest role in separating the two groups and applied them to PD subjects taking dopaminergic medications. Using these parameters, they achieved a complete separation of the PD patients from controls. 8-OHdG levels were significantly increased in PD patients, but overlapped controls. Two other markers of oxidative damage were measured in their LCECA profiles. Uric acid was significantly reduced while glutathione was significantly increased in PD patients. These findings show that metabolomic profiling with LCECA holds great promise for developing biomarkers for both the diagnosis, as well as monitoring disease progression in PD. It may lead to the development of the first accurate diagnostic test for PD.

Protein Biomarkers of PD

Proteins in serum have been proposed to be informative regarding what disease pathways and mechanisms of neuronal degeneration are active in patients (Goldknopf 2008). An inflammatory process may manifest in PD serum and particularly in men with high plasma concentrations of IL-6 may have an increased risk of developing PD (Chen et al. 2008). α-synuclein, detected by proteomic technologies, is a peripheral biomarker of PD (Jesse et al. 2009). α-synuclein is also found in platelets and skin biopsies of PD patients. Increased α-synuclein expression is known to cause a rare form of PD, early-onset autosomal dominant PARK4. The induction of α-synuclein expression was found to constitute a specific disease biomarker in sporadic PD patient fibroblasts. Loss-of-function mutations of the mitochondrial kinase PINK1, which cause the early-onset recessive PARK6 variant result in oxidative damage in fibroblasts of PD patients by enhanced α-synuclein expression and altered cell-cell contact (Hoepken et al. 2008). α-synuclein induction might represent a common event for different variants of PD as well as a PD-specific trigger of neurodegeneration, and serve as a potential biomarker for objective diagnosis of PD in an accessible, peripheral tissue.

P11 Protein as a Biomarker of Depression in PD

Depression affectsup to 40% of patients with PD during their lifetime adversely affecting the quality of life and is associated motor and cognitive decline. It can be a prodromal or preclinical feature up to 10 years before diagnosis of PD.

Altered expression of p11 protein in PD has been analyzed using postmortem brain tissue and antemortem peripheral blood from PD patients classified as depressed or nondepressed (Green et al. 2017). Important findings of this study were:

- P11 levels were found to be significantly lower in PD patients compared with controls not only in the putamen and substantia nigra, regions of the midbrain nigrostristal pathway most affected by PD pathology, but also in the cerebral cortex.
- Measured of p11 protein in peripheral blood leukocytes from peripheral blood mononuclear cells of PD patients in advanced stage revealed significant positive correlations between p11 expression in monocytes with total UPDRS score as well as Hoehn and Yahr staging in all PD patients.
- P11 levels in CD8+ cells showed the best diagnostic characteristic with sensitivity of 87% and specificity of 93%.

Important comments in a review of the study of Green et al. are (Mollenhauer and Weintraub 2017):

- The findings of this impressive study need replication, particularly in less-advanced PD subjects.
- A possible cellular link between p11 and the immune system is significant in the context of depression, other PD symptoms and inflammation.
- Limitations of the study include not conducting secondary analyses excluding antidepressant-treated patients, because nearly half of depressed patients were taking an antidepressant, which have effects on p11.
- Lack of control in correlation analyses of UPDRS score or Hoehn and Yahr stage in depression, given the association between these variables themselves and each with p11.
- Role of p11 in PD pathogenesis and neurodegeneration of dopamine and other neurons still needs to be elucidated, but if this finding is replicated by further studies, p11 could serve as a biomarker for PD phenotype and progression.

Serum Vitamin D as a Biomarker of PD

A longitudinal study from Finland has demonstrated an association between low serum vitamin D levels and the subsequent development of PD (Knekt et al. 2010). In the 29-year follow-up of 3173 subjects who were free from PD at start of the study, serum 25-hydroxyvitamin D level was determined from frozen samples stored at baseline. Estimates of the relationship between serum vitamin D concentration and PD incidence were calculated using the Cox model. Individuals with higher serum vitamin D concentrations showed a reduced risk of PD.

In another study on a cohort of untreated patients with early PD, prevalence of vitamin D insufficiency (25-hydroxyvitamin D concentration < 30.0 ng/mL) was 69.4% at baseline and 51.6% at the end point (Evatt et al. 2011). The prevalence of

vitamin D insufficiency in patients with early PD was similar to or higher than those reported in previous studies. Vitamin D concentrations did not decline during progression of PD. Further studies are needed to elucidate the natural history and significance of vitamin D insufficiency in PD.

The mechanisms of vitamin D's neuroprotective effect in PD are not understood, but it may act via antioxidative mechanisms, immunomodulation, or enhanced nerve conduction. Vitamin D receptors and an enzyme responsible for the formation of the active form, 1,25-hydroxyvitamin D, have been found in high levels in the substantia nigra, the region of the brain affected most in PD. This raises the possibility that chronic inadequacy of vitamin D leads to the loss of dopaminergic neurons in substantia nigra.

Biomarkers of Prodromal PD

The term "prodromal PD" refers to individuals who do not fulfill motor diagnostic criteria for PD, but who have clinical, genetic, or biomarker characteristics suggesting risk of developing PD in the future. Clinical diagnosis of prodromal PD has low specificity, prompting the need for objective biomarkers with higher specificity. In this qualitative review, we discuss objectively defined putative biomarkers for PD and prodromal PD.

Future Needs for Biomarkers of PD

Considerable progress has been made during the past two decades in identifying and assessing PD biomarkers, but no fully validated biomarker for PD is yet available. There is still no objective endpoint for clinical trials for early PD other than the Unified PD Rating Scale, which is a rater-based assessment of symptom severity. There is a need for objective biomarkers that are present in persons at-risk of PD, are neuropathophysiological manifestations of the disease, or track the rate of its progression.

Several efforts are being made for discovery of better biomarkers of this disease. In view of the multiple genetic causes for PD that have already been identified, the marked variability in the loss of dopaminergic biomarkers measured by imaging at the time of onset of motor symptoms and the heterogeneity of clinical symptoms at onset and during clinical progression, it is likely that multiple biomarkers encompassing pathobiology to molecular genetic mechanisms will be necessary to fully map risk and progression of PD. Detection of biomarkers of PD before clinical onset will be a further challenge. Proteomic technologies are promising for selection and overview of potential protein biomarkers of PD. A combination of multiple or at least two proteins will be necessary to differentiate PD from dementia from other dementing syndromes. A systems biology approach and use of microarrays

are promising for discovery and evaluation of biomarkers of PD. Biomarkers for detecting the early stages of PD could identify individuals at risk for developing the disease, to improve early diagnosis, to track disease progression with precision, and to test the efficacy of new treatments.

Parkinson's Progression Markers Initiative (www.ppmi-info.org) is a longitudinal study of PD patients with early disease and age-matched healthy individuals as controls to identify biomarkers of PD progression (Sherer 2011). Recruitment uses highly standardized clinical, neuroimaging, and biological protocols. Clinical data collection includes motor assessments, neuropsychiatric/cognitive testing, olfaction, DaTSCAN imaging and MRI. Lead biological candidates to be tested include: α-synuclein (CSF), DJ-1 (CSF and blood), urate (blood), $A\beta_{1-42}$ (CSF), total tau, and phospho-tau (CSF).

H25K microarray MAbs generated against native human plasma proteins and PlasmaScan™ 80 technologies (Arrayit Corporation) have enabled rapid and efficient plasma sample preparation of specimens from well-characterized PD patients for discovery of biomarkers. There is hope that biomarkers will help diagnose symptomatic and presymptomatic PD or provide surrogate endpoints to demonstrate clinical efficacy of neuroprotective therapies in clinical trials, and help stratify this heterogeneous disease. No single biomarker is likely to fulfill all these functions, so there is a need to know how each has been validated in order to understand their uses and limitations, and be aware of potential pitfalls. Ultimately, neuroprotective studies will require accurate surrogate biomarkers of disease progression that correlate with clinical improvement.

Biomarkers of Huntington's Disease

Huntington's disease (HD) is an autosomal dominant disorder caused by an expansion of glutamine repeats in ubiquitously distributed huntingtin protein. It is characterized clinically by progressive motor impairment, cognitive decline, and various psychiatric symptoms, with the typical age of onset in the third to fifth decades. Genetic testing for the disease can be performed to identify presymptomatic mutation carriers, providing a unique opportunity for researchers to use brain imaging to track the neurodegenerative process. Measurement of the number of cytosine, adenine, and guanine (CAG) repeats in the HD gene represents an effective, direct test with which to confirm the clinical diagnosis in difficult cases. Although a genetic test is available as a trait marker, biomarkers of the disease state are still in development. Mutant huntingtin interferes with the function of widely expressed transcription factors, suggesting that gene expression may be altered in a variety of tissues in HD, including peripheral blood. Use of microarrays to analyze global gene expression in blood samples of HD patients has identified several mRNAs with significantly altered expression in HD compared with controls. By this approach it is possible to distinguish presymptomatic individuals carrying the HD mutation from symptomatic HD patients. Expression of the biomarker genes is significantly

Table 14.6 Biomarkers of Huntington disease

CAG length: diagnostic biomarker
Mutant huntingtin protein
Neuroimaging biomarkers
Biochemical: magnetic resonance spectroscopy
Functional neuroimaging: PET, SPECT, fMRI
Metabolic biomarkers detected by brain imaging
CSF biomarkers
Monoamine metabolites
Tryptophan pathway metabolites
F2-isoprostanes, a marker of lipid peroxidation and oxidative stress
Measures of transglutaminase activity
Biomarkers in plasma
Amino acids decreased: valine, leucine, and isoleucine
Blood cells: transcriptional biomarkers
miR-34b
Oxidative stress, cell death pathways, inflammation, proteolysis, etc
Plasma: metabolomic profiling of small molecules

up-regulated in postmortem HD caudate nucelus, suggesting that alterations in blood mRNAs may reflect disease mechanisms observed in HD brain. Thus, changes in blood mRNAs can distinguish HD patients from controls. These alterations in mRNA expression correlate with disease progression and response to experimental treatment. Such biomarkers may provide clues to the state of HD and may be of predictive value in clinical trials. Identification of a reliable and robust biomarker in HD disease is a growing area of research, with particular importance for future studies of disease-modifying therapies (Ross et al. 2014). A list of biomarkers of HD is shown in Table 14.6.

A significant decrease in the plasma branched-chain amino acids valine, leucine, and isoleucine has been shown in HD individuals, compared with controls (Mochel et al. 2011). miR-34b is elevated in plasma from HD gene carriers prior to the onset of clinical symptoms, and is a potential a plasma biomarker for HD (Gaughwin et al. 2011).

Measurement of huntingtin is very important as several potential therapies for HD are targeted against either huntingtin itself or against some of its functional properties. Measuring huntingtin levels, its physical state, its proteolytic products, or its interactions with other molecules would all have distinct values as biomarkers, especially as pharmacodynamic indicators (Hersch and Rosas 2011).

Glutamate-mediated excitotoxicity is presumably pivotal in HD pathogenesis and explains the beneficial effect of creatine therapy, which lowers glutamate levels in the cerebral cortex. Serum 8-hydroxy-2′-deoxyguanosine levels are markedly elevated in HD as an indicator of oxidative injury to DNA, and are reduced by creatine treatment.

Elevated levels of iron have been observed in the caudate nucleus and putamen in early HD individuals, compared to presymptomatic mutation-positive and control individuals (Dumas et al. 2012). MRI susceptibility and MRS studies observe progressive increases in iron and other transition metals compared to controls in presymptomatic mutation carriers and motor symptomatic HD (van Bergen et al. 2016). These studies imply that changes in iron homeostasis could occur very early in HD pathogenesis.

Genetic Biomarker of HD Progression

The gene MSH3, a DNA repair gene, has been extensively implicated in the pathogenesis of HD in both mouse and cell studies. A research team has used the high quality phenotypic data from the intensively studied TRACK-HD cohort of persons with the HD gene mutation and looked for areas of the genome associated with their progression and validated the findings in a larger sample of from a separate cohort, the European Huntington's Disease Network REGISTRY study (Moss et al. 2017). The genetic signal is likely driven by the gene MSH3, that has been linked to changes in size of the HD mutation, and the researchers identified a variation in MSH3 that encodes an amino acid change in the gene. This genetic biomarker of HD progression is a potential therapeutic target for HD as MSH3 knockout in animal models of HD reduces progression of HD.

Quantitative MRI Measurement of Brain Atrophy as Biomarker of HD

Changes in brain atrophy in subjects with HD have been quantified by quantitatively measured the rate of brain atrophy by MRI as fractional gray matter (GM), white matter (WM), and corresponding CSF in gene-positive subjects with presymptomatic to advanced HD, and age-matched healthy controls (Squitieri et al. 2009). Each of the three brain compartments had a diverse role and their time courses differed in the development of HD. GM volume decreased early in life associated with decreased serum BDNF and started even many years before onset of symptoms, then decreased slowly in a nonlinear manner during the various symptomatic HD stages. WM volume loss also began in the presymptomatic stage of HD a few years before manifest symptoms appear, rapidly decreasing near to the

zone-of-onset. Finally, the CSF volume increase began many years before age at onset. Its volume measured in presymptomatic subjects contributed to improve the CAG-based model of age at onset prediction. The progressive CSF increase depended on CAG mutation size and continued linearly until the last stages of HD, perhaps representing the best biomarker of progression rate and severity in HD. A combined neuroimaging approach incorporating structural MRI, fMRI and diffusion tensor (DT)-MRI is yet to be realized in HD clinical trials, but if proven to be sensitive and reliable, it could potentially provide a biomarkers for use in future clinical drug trials in HD (Bohanna et al. 2008).

Metabolic Networks as Biomarkers of Preclinical Huntington Disease

A computational approach has been used to identify a functional brain network associated with the progression of preclinical HD. Progressive metabolic abnormalities involving a specific network of disease regions were identified on ^{18F}FDG PET scans in premanifest HD mutation carriers to measure network-wide changes in cerebral metabolic activity over a 7-year period (Tang et al. 2013). The rate of preclinical progression measured for the network as verified in a separate cohort of premanifest HD carriers was faster, ie, progressed at approximately twice the rate than conventional single-region estimates from the same subjects. Thus metabolic network measurements provide a sensitive means of quantitatively evaluating disease progression in premanifest individuals. This approach may be incorporated into clinical trials to assess disease-modifying agents.

Biomarkers of Wilson's Disease

Wilson's disease is a disorder of copper metabolism and affects approximately 1 in 20,000 persons worldwide. It is an autosomal recessive disorder caused by defects in the ATPase, Cu^{2+} transporting, β-polypeptide gene (ATP7β) resulting in accumulation of copper in liver and brain. Approximately half of Wilson's disease patients are never diagnosed and die of untreated disease. The disease can be thwarted if detected at a presymptomatic stage and copper levels can be reduced by administration of penicillamine. One reason for this lack of diagnosis is the fact that there is currently no single test for the disease. Currently, a combination of symptoms, laboratory testing and liver biopsy is used to make the diagnosis. SNP biomarkers can be used in combination with analysis of prevalent mutations as a comprehensive strategy for determining presymptomatic and carrier siblings of Wilson's disease patients (Gupta et al. 2007).

An assay has been developed for quantifying ceruloplasmin (CP), the biomarker for Wilson disease, in dried blood spots (DBS) for newborn screening of Wilson disease (deWilde et al. 2008). CP-specific peptides from DBS samples digested by trypsin were quantified using isotopically labeled peptide internal standards and LC-MS/MS. Results of this study supports that newborn screening for ceruloplasmin is feasible.

Biomarkers of Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is progressive, devastating syndrome that affects both upper and motor neurons. It results in limbs and facial motor weakness, atrophy, spasticity, and death. It is referred to as motor neuron disease in UK and some other European countries and as Charcot's disease in France. It is also referred to as Lou Gehrig's disease in the US. A number of biochemical disturbances have been identified to explain the pathomechanisms of ALS. There is some evidence that initial susceptibility to ALS is conferred through a genetic though not necessarily an inherited defect. Several genetic defects have been identified. A familial form of ALS is also known.

The only specific diagnosis of sporadic ALS is neuropathologic, i.e., the presence of inclusions staining positively for ubiquitin and TAR DNA-binding protein in degenerating motor neurons. Currently, there is a delay of about 1 year from onset of symptoms to clinical diagnosis; at least 30% of anterior horn neurons are degenerated by the time distal muscle wasting is visible. Therefore, reliance on symptoms or clinical examination to trigger intervention might not be adequate if degeneration is no longer salvageable at that stage. Thus, an early diagnostic biomarker might prove to be clinically useful only if those at risk of developing ALS can be identified and screened before the onset of symptoms. As there are no diagnostic biomarkers for ALS, this uncertainty hinders development of therapy, health care coverage, categorization of candidate patients, and care planning. Early diagnosis will affect the disease accommodation, patient care planning, and identification of the appropriate treatment regime. Table 14.7 shows classification of biomarkers of ALS.

ALS Biomarker Detection in Blood Versus CSF

As in the case of other CNS biomarkers, CSF is a better source than blood. Nevertheless, search for biomarkers in blood continues as it is a non-invasive method. Several studies have reported candidate biomarkers of ALS in blood but most of these studies were flawed. Aminoaciduria and raised concentrations of tyrosine as well as glutamate have been observed in the serum in some ALS patients. Among cytokine biomarkers, raised concentrations of IL-6 have been shown in the

Table 14.7 Classification of biomarkers of amyotrophic lateral sclerosis

Biomarkers of neuroinflammation
Biomarkers of axonal damage
Tau protein
Neurofilaments (NfHSMI35)
Genetic biomarkers
C9orf72 (chromosome 9 open reading frame 72) gene mutation
DAO (D-amino-acid oxidase)
FUS (fused in sarcoma) gene mutation
OPTN (optineurin)
SOD1 (superoxide dismutase 1) gene mutation
TARDBP (TAR DNA binding protein)g ene mutations
VCP (valosin-containing protein)
Imaging biomarkers of ALS
NMR spectroscopy, DT-MRI
Metabolomic biomarkers
Neurophysiological biomarkers of ALS
Biomarkers of upper motor neuron lesions: decrease in duration of the cortical silent period
Biomarkers of lower motor neuron lesions: axonal hyperexcitability, loss of motor units
Proteomic biomarkers
Antibodies that recognize a disease-specific ALS-related protein
Cystatin C
Peptic fragment of neurosecretory protein VGF

serum of patients with ALS. Because oxidative stress as an important factor in the pathogenesis of ALS, serum concentrations of 4-hydroxy-2,3-nonenal, a toxic product of lipid peroxidation, and monocyte chemoattractant protein-1α, an inflammatory marker, have been investigated and found to be elevated. Of all the plasma biomarkers of ALS, the most promising are the proteomic biomarkers as described in a later section.

CSF biomarkers, despite the lumbar puncture required to obtain the specimen, have a better chance to detect ALS at an earlier stage before neuronal damage occurs. There are >65 studies reporting biomarkers of ALS in CSF. Raised levels of glutamate have been found in CSF parallel to studies of raised levels of this excitatory amino acid in the blood. Reduced levels of various neurotrophic factors have also been observed. Increased levels of substance P and decreased levels of erythropoietin have also been reported. Several CSF protein biomarkers are described in the section on proteomics.

Biomarkers of Neuroinflammation in ALS

A CSF inflammatory profile associated with ALS pathogenesis has been described that may distinguish patients with ALS from neurologic disease controls, and may serve as a biomarker panel to aid in the diagnosis of ALS pending further validation (Mitchell et al. 2009). Fourteen biomarkers differed between patients with ALS and the control group. The five proteins with the lowest p values differentiated patients with ALS from controls with 89.2% accuracy, 87.5% sensitivity, and 91.2% specificity. Expression of IL-8 was higher in those patients with lower levels of physical function. Expression of β2-microglobulin was higher in subjects carrying an H63D HFE allele, while expression of several biomarkers was higher in subjects carrying a C282Y HFE allele.

Genetic Biomarkers of ALS

Investigations of the 10% of ALS cases that are transmitted as dominant traits have revealed numerous gene mutations and variants that either cause these disorders or influence their clinical phenotype. Gene mutations in ALS may cause disturbances of protein homeostasis, alterations in RNA binding proteins, and defects in cytoskeletal dynamics, as well as numerous downstream pathophysiological events (Ghasemi and Brown 2017). Major genes underlying ALS are SOD1, TARDBP, FUS, OPTN, VCP, UBQLN2, and C9ORF72.

One of the most frequently affected gene is that for superoxide dismutase (SOD) 1, which is a powerful antioxidant enzyme. Most commonly identified mutations in SOD1 that affect protein activity are D90A, A4V and G93A, which lead to the accumulation of highly toxic hydroxyl radicals (Kaur et al. 2016). Accumulation of free radicals causes degradation of both nuclear and mitochondrial DNA as well as protein misfolding – pathological feature of ALS. Disease specific TDP-43 variants are found in sporadic as well as familial cases of ALS and a small panel of single chain antibody fragment that recognize these variants can generate a neuropathological and plasma biomarker profile to distinguish different TDP-43 pathologies (Williams et al. 2017). Massive hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS and frontotemporal dementia (FTD) and accounts for ~40% of cases of familial ALS and genetically explains the majority of the overlap of ALS and FTD (Renton et al. 2014).

Imaging Biomarkers of ALS

A number of studies in ALS patients have shown that magnetic resonance spectroscopy (MRS) and diffusion tensor (DT)-MRI can detect corticospinal lesions. MRS reveals reduction of N acetyl aspartate (NAA), a biomarker of neuronal integrity.

In ALS, decreased NAA is not only restricted to the motor cortex and corticospinal tract, but is also observed in premotor regions, the primary sensory cortex, and extramotor frontal regions with relative sparing of the parietal lobe. However, because of their relative lack of sensitivity and specificity, these techniques are currently inadequate for use as diagnostic tools in individual patients.

DT-MRI reveals the structure of nerve fibers in the corticospinal tract that correlates with function, thus revealing the ALS pathology. DT tractography enables visualization and evaluation of corticospinal and corticobulbar tract dysfunction in patients with ALS. In vivo DT-MRI measures demonstrate differences in white matter degeneration between sporadic ALS and a unique familial form of the disease, indicating that genotype influences the distribution of cerebral pathologic features in ALS (Stanton et al. 2009). Functional MRI (fMRI) reveals a general pattern of cortical reorganization for motor function in patients with ALS when compared with that seen in normal subjects.

In spite of limitations, imaging modalities provide insight into the pathophysiological process in ALS and may play a role in accurate identification of upper motor neuron pathology in ALS. Further investigations are needed to determine and to compare the relative usefulness of various neuroimaging techniques (Wang et al. 2011).

Metabolomic Biomarkers of ALS

Some metabolic biomarkers are consistently found in patients with ALS and can be used to monitor the course of ALS in patients taking the drug riluzole. These biomarkers are not only useful for diagnostics and patient stratification but can also be mapped on a biochemical chart to identify the corresponding enzyme for target identification.

The metabolites that are significantly up and down regulated in ALS constitute a preliminary biochemical signature for the disease. The specificity of these signatures will be further validated by comparison to metabolic profiles in other neurodegenerative diseases, in neuromuscular disorders. If these signatures can be confirmed in a large population of patients just after diagnosis or through retrospective analysis of preexisting banked sera, these signatures would become diagnostic markers for the disease. Such biomarkers, once their chemical identities are determined and mapped to biochemical pathways, may point to new hypotheses about the pathogenesis of the disease.

Proteomic Biomarkers of ALS

Proteomic analysis can identify protein biomarkers that provide an insight into disease pathogenesis and are useful for diagnosis. To identify ALS specific biomarkers, proteomic profile of CSF from ALS and control subjects were compared using SELDI-TOF-MS. Three protein species (4.8-, 6.7-, and 13.4-kDa) that were

significantly lower in concentration in the CSF from patients with ALS than in normal controls were identified (Pasinetti et al. 2006). A combination of three protein species (the "three-protein" model) correctly identified patients with ALS with 95% accuracy, 91% sensitivity, and 97% specificity from the controls. Independent validation studies using separate cohorts of ALS, healthy control, and peripheral neuropathy subjects confirmed the ability of the three CSF protein species to separate patients with ALS from other diseases. Protein sequence analysis identified the 13.4-kDa protein species as cystatin C and the 4.8-kDa protein species as a peptic fragment of the neurosecretory protein VGF.

CSF levels of tau protein and neurofilaments (NfHSMI35), biomarkers of axonal damage,were five times higher in patients with ALS (1.7 ng/mL) than in controls as measured by ELISA (Brettschneider et al. 2006). Values of NfHSMI35 were higher in ALS of more rapid progression. The values of NfH and tau did not correlate with CSF protein content. Biomarkers of axonal damage in the CSF may discriminate between subtypes of ALS and could be used as biomarkers for therapeutic trials. CSF NfH is superior to tau in these discriminations. In CSF specimens, MS peaks for cystatin C and transthyretin are reduced in ALS, whereas peaks for posttranslational modified transthyretin and CRP are increased predicting ALS with an overall accuracy of 82% (Ryberg et al. 2010). Measurement of galectin-3 in CSF samples showed that ALS patients had approximately twice as much galectin-3 as normal and disease controls (Zhou et al. 2010).

Ideal Biomarker of ALS

The ideal characteristics of an ideal biomarker of ALS are:

- Sensitivity and specificity for ALS, ideally prior to the onset of substantial wasting or weakness, or even at the presymptomatic stage for rare familial forms.
- Ability to reliably discriminate between clinical phenotypes with prolonged survival at symptom onset or even earlier.
- Ability to predict regional involvement and the pattern of spread in advance of symptoms, which would permit prophylactic intervention for supportive care.
- Ability to change in a predictable way with progression of disease and with sufficient sensitivity to permit confident judgment of therapeutic response within days to weeks of therapeutic challenge.
- Affordable technology as well as easy accessibility and measurements even in patients with physically disability.

Future of Biomarkers of ALS

Potential biomarkers that are sensitive to the progression of disease, which might enhance the diagnostic algorithm and provide new drug targets, are now being identified from analysis of the blood and CSF, as well as from neuroimaging and

neurophysiology studies. In combination, these biomarkers might be sensitive to early therapeutic effects and would reduce our reliance on animal models, which have uncertain relevance to sporadic ALS in human beings. Such biomarkers might also resolve complexities of phenotypic heterogeneity in clinical trials. In the future, a panel of biologic, genetic, radiologic, and electrophysiologic biomarkers, rather than a single biomarker, may prove to be useful for the diagnosis and follow-up of ALS patients.

HIV-1-Associated Neurocognitive Disorders

CNS is an important reservoir for HIV. HIV-1-associated neurocognitive disorders (HAND) persist in infected individuals despite adequate immunological status. Risk factors for cognitive impairment include hepatitis C virus co-infection, host genetic factors predisposing to HAND, the early establishment of the virus in the CNS and its persistence under antiretroviral therapy. NLRP3 inflammasome-associated genes are expressed in the brains of HIV-1-infected persons and contribute to encephalopathy. Inflammasomes can be triggered by alarmins or danger-associated molecular patterns (DAMPs), which directly stimulate the production of proinflammatory mediators by glial cells, contribute to BBB through release of various proteases, allow the passage of infected macrophages, and trigger IL-1β release from primed cells. Amongst alarmins involved in HIV-1-induced neuropathogenesis, IL-33 and high-mobility group box 1 (HMGB1), which plays a crucial role in HIV-1 persistence in DCs, are of particular interest. Neurocognitive alterations have been associated with dysregulation of the IL-33/ST2 axis in the CNS, leading to the induction of neuronal apoptosis, decrease in synaptic function and neuroinflammation. Specific biomarkers, including HMGB1 and anti-HMGB1 antibodies, have been identified in CSF from patients with HAND, correlated with immune activation and identifying a very early stage of neurocognitive impairment that precedes changes in metabolites detected by MRS (Gougeon 2017).

Biomarkers of Dementia in HIV-1-Infected Patients

In order to identify biomarkers that are associated with and can predict dementia in HIV-1 infected patients, sphingolipid, sterol, triglyceride, antioxidant, and lipid peroxidation levels in CSF were analyzed and changes in cognitive status were recorded over a 1-year period (Bandaru et al. 2007). Increased levels of the vitamin E and triglyceride C52 predicted the onset or worsening of dementia. Elevated levels of sphingomyelin were associated with inactive dementia. Elevated levels of ceramide and the accumulation of 4-hydroxynonenals were associated with active dementia.

These findings indicate that early in the pathogenesis of HIV dementia, there is an upregulation of endogenous antioxidant defenses in brain. The failure of this attempted neuroprotective mechanism leads to the accumulation of sphingomyelin and moderate cognitive dysfunction. The breakdown of this enlarged pool of sphingomyelin to ceramide and the accumulation of highly reactive aldehydes are associated with declining cognitive function. Thus, elevations in endogenous protective mechanisms may identify patients who are at increased risk of the development of HIV dementia.

Biomarkers of Autoimmune Encephalitis

Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is a severe but treatable autoimmune encephalitis affecting mainly young adults and children. The lack of suitable biomarkers of disease activity makes treatment decisions and identification of relapses challenging. Concentration of CXCL13 was determined with ELISA in all available patients' samples (serum and CSF) in a retrospective cohort study of patients consecutively diagnosed as having anti-NMDAR encephalitis (Leypoldt et al. 2015) Seventy percent of patients with early-stage anti-NMDAR encephalitis had increased CSF CXCL13 concentration that correlated with intrathecal NMDAR-antibody synthesis. Prolonged or secondary elevation of CXCL13 was associated with limited response to treatment and relapses. Thus CXCL13 is a potentially useful biomarker of treatment response and outcome in anti-NMDAR encephalitis.

Biomarkers of Prion Diseases

Protein misfolding is implied in prion diseases. The prion protein (PrP) is a widely expressed, membrane-associated protein transcribed from the PRNP gene, which is highly conserved among mammals. PrPs exist in two forms: a common, harmless α-helical form, and a rare β-sheet form that causes fatal mammalian brain diseases such as scrapie, Creutzfeld-Jakob disease (CJD), and bovine spongiform encephalopathy (BSE) or mad cow disease. Prion diseases collectively are called transmissible spongiform encephalopathies (TSEs), causing sponge-like vacuoles in the brain. Symptoms include dementia and ataxia. The principal component of prions is the glycoprotein PrPSc, which is a conformationally modified isoform of a normal cell-surface protein called PrPC. During the time between infection and the appearance of the clinical symptoms, minute amounts of PrP^{Sc} replicate by conversion of host PrPC, generating large amounts of misfolded prion protein (PrP^{Sc}) aggregates in the brains of diseased individuals.

14-3-3 Protein and tTau/P-Tau Ratio

Due to the few specific premortal diagnostic signs, it is difficult to separate CJD clinically from rapidly progressing AD and other rapidly progressing neurological diseases. As TSEs can cross species barriers, there is a public health concern about the ability of TSE to spread from other species to humans. Definite diagnosis of CJD is established only by brain biopsy and there is need for a less invasive diagnostic procedure. The total concentration of tau protein (tTau) in CSF has been found to separate patients with CJD from those with AD. As an increased concentration of tTau is regarded to be a biomarker for degradation of neurons, the success of tTau as a biomarker for CJD depends on whether other neurological diseases have the same high amount and rate of neuronal degeneration as CJD. A study assessed the utility of tTau, the ratio of (tTau)/181 phosphorylated tau protein (P-Tau) and 14-3-3 protein, as diagnostic biomarkers in CSF in CJD (Skinningsrud et al. 2008). The results suggest that 14-3-3 protein may be a better biomarker for CJD than tTau/P-Tau ratio and tTau, which are also efficient biomarkers, but showed slightly inferior diagnostic properties in this study, with tTau/P-Tau marginally better than tTau.

Bioluminescence Imaging as a Surrogate Biomarker of Prion Infectivity

Because astrocytic gliosis marked by the deposition of fibrils composed of GFAP is a prominent feature of prion disease, it has been considered as a surrogate biomarker for prions. To investigate this hypothesis, prions were inoculated into transgenic (Tg) mice expressing luciferase (luc) under the GFAP gene (Gfap) promoter, denoted Tg(Gfap-luc) mice (Tamgüney et al. 2009). Weekly noninvasive, bioluminescence imaging (BLI) detected an increase in light emitted from the brains of Tg(Gfap-luc) mice at ≈55 d after inoculation and ≈62 d before neurologic deficits appeared. Endpoint titrations of prions were also performed in Tg(Gfap-luc) mice. BLI bioassays were as or more sensitive than those determined by the onset of neurological dysfunction, and were completed in approximately half the time. These studies indicate that BLI is likely to be a suitable surrogate biomarker for measuring prion infectivity, and might be useful in the study of Tg mouse models for other neurodegenerative illnesses.

miRNAs as Biomarkers of Prion-Induced Neurodegeneration

A number of genes and signaling pathways important in neuronal degeneration in prion diseases are likely regulated at least in part by miRNAs. Several miRNAs that appear to be de-regulated during prion-induced neurodegeneration have been identified. A co-ordinated de-regulation of miRNAs seen in prion diseases may well be

a response to the abnormal accumulation of PrP^{Sc} leading to a pathogenic cascade consisting of compensatory alterations in neurotransmitter receptors, protein degradation pathways and signaling pathways which lead to an ultimate failure of neuronal function. MiRNAs also have potential for use as biomarkers given the evidence of de-regulation of a prion-specific subset of miRNAs that is distinct from other neurodegenerative diseases (Saba et al. 2008). Given the ability of miRNAs to target multiple genes for expression, and their potential for modulating neuroprotective mechanisms, there is a potential for miRNAs to be used as therapeutics in neurodegenerative diseases.

Prion Protein Detection by Real-Time Quaking-Induced Conversion

An in vitro test can detect the specific biomarker in CSF for CJD, the prion protein (PrP^{CJD}), by means of real-time quaking-induced conversion (RT-QuIC) – an ultrasensitive, multiwell plate-based fluorescence assay involving PrP^{CJD}-seeded polymerization of recombinant PrP into amyloid fibrils. The testing has a sensitivity of 80 to 90% for the diagnosis of sporadic CJD. In a preliminary study, RT-QuIC testing of olfactory epithelium samples obtained from nasal brushings was accurate in diagnosing CJD and indicated substantial prion seeding activity lining the nasal vault (Orrú et al. 2014). Nasal brushings elicited stronger and faster RT-QuIC responses than CSF for the between-group comparison of strength of response. Individual brushings contained ~10^5–10^7 prion seeds, at concentrations several $\log_{10}$ greater than in CSF.

Prions in the Urine of Patients with Variant CJD

To investigate whether PrP^{Sc} can be detected in the urine of patients with variant CJD, the protein misfolding cyclic amplification (PMCA) technique was used to amplify minute quantities of PrP^{Sc}, enabling highly sensitive detection of the protein (Moda et al. 2014). PrP^{Sc} was detectable only in the urine of patients with variant CJD and had the typical electrophoretic profile associated with this disease. PrP^{Sc} concentration in urine calculated by means of quantitative PMCA was estimated at 1×10^{-16} g/mL or 3×10^{-21} mol/mL, which extrapolates to ~40 to 100 oligomeric particles of PrP^{Sc} /mL of urine.

Biomarkers of Multiple Sclerosis

Multiple sclerosis is a complex disease, as several pathophysiological processes (including inflammation, demyelination, axonal damage, neurodegeneration and repair mechanisms) participate in the disease process. Corresponding to these,

several proteins are under investigation: e.g. neurofilaments, tau, 14-3-3 proteins, and N-acetylaspartate and Nogo-A. Furthermore, as new pathological evidence reveals, these processes are not uniformly represented across patient populations but can selectively predominate in individual patients, thus contributing to the heterogeneity in phenotypic expression of the disease, its prognosis and response to therapies. However, there are limited biomarkers of multiple sclerosis in serum. CSF, which is more likely to reflect the disease, is obtainable by lumbar puncture only and not suitable for general screening or monitoring therapy. Biomarkers of multiple sclerosis are listed in Table 14.8.

Antibodies in Multiple Sclerosis

There is an increased frequency of several antibodies in patients with multiple sclerosis. Antiphospholipid antibody positivity in multiple sclerosis patients varies between 2% and 60%. Anticardiolipin antibody can be detected in CSF in multiple sclerosis and other CNS disorders including systemic lupus erythematosus and AIDS. Although the presence of these antibodies is probably an epiphenomenon of disseminated immunological dysfunction in multiple sclerosis, an atypical disease course is often suspected in patients with positive autoantibodies. Multiple sclerosis patients with positive anticardiolipin antibody may have increased incidence of myelopathy and optic neuritis even though MRI of the brain may be normal. The frequency of antinuclear antibody is also increased in multiple sclerosis patients; the prevalence is variable, ranging between 2% to 26%. There may be a positive correlation between antinuclear antibody level and persistency and disease activity. However, no correlation with age, disease course, and disability is found. Increased titers of anti-thyroglobulin and anti-peroxidase antibodies are detected in a significantly higher percentage of multiple sclerosis patients. These antibodies occur early in the disease course and may have a protective role.

Increased titers of myelin-specific antibodies in serum and CSF are frequently observed in multiple sclerosis patients. Anti-myelin oligodendrocyte glycoprotein antibodies may be present in active lesions in patients with multiple sclerosis. A substantial proportion of multiple sclerosis patients show a persistent autoantibody response to myelin oligodendrocyte glycoprotein. The presence of serum anti-myelin oligodendrocyte glycoprotein and anti-myelin basic protein antibodies in patients with an initial event suggestive of CNS demyelination and lesions on MRI may be predictive of subsequent development of clinically definite multiple sclerosis.

Antibodies to Galactocerebroside

Galactocerebroside, the major glycolipid of CNS myelin, is a known target for pathogenic demyelinating antibody responses in experimental allergic encephalomyelitis (EAE), the animal model of multiple sclerosis. There are significant

Table 14.8 Biomarkers of multiple sclerosis

Antibodies
Antibodies to galactocerebroside in serum
Antibodies to myelin oligodendrocyte glycoprotein in CSF only
Anticardiolipin antibody in both serum and CSF
Antinuclear antibody in both serum and CSF
Antithyroid antibody in serum only
Myelin associated glycoprotein in both serum and CSF
Myelin basic protein in serum as well as CSF
Proteolipid protein in CSF
Switch-associated protein 70 antibodies in serum
Biomarkers reflecting alteration of the immune system
Adhesion molecules
Biomarkers reflecting immune-mediated neuroprotection
Biomarkers reflective of antigen processing and presentation
Cell cycle and apoptosis-related biomarkers
Chemokines and their receptors
Complement-related biomarkers
Cytokines and their receptors
Oligoclonal bands
T cells: migration into the CNS, T cells as biomarkers of response to treatment
Biomarkers in CSF
Biomarkers of myelin destruction in CSF
Cystatin
Neurofilaments
Biomarkers of blood-brain barrier (BBB) disruption
Matrix metalloproteinases
Biomarkers of oxidative stress
Biomarkers of axonal/neuronal damage
Biomarkers of gliosis
Glial fibrillary acidic protein
S100B
Biomarkers of remyelination and repair
Biomarkers of response to therapy and prognosis
DNA motifs in the blood as biomarkers of response to treatment
Gene expression
Lymphocyte subpopulations
Vitamin D
Molecular imaging biomarkers

differences in serum alpha-GalC IgG titers between patients with relapsing-remitting (RR)-multiple sclerosis and healthy controls has been reported. Alpha-GalC antibodies appear multiple sclerosis-specific and are not found in healthy subjects, unlike antibodies against myelin proteins; when present, alpha-GalC antibodies identify mostly RR-multiple sclerosis and may be an indicator of ongoing disease activity. This novel assay is a suitable and valuable method to increase accuracy of diagnosis and disease staging in multiple sclerosis.

Antibodies to Myelin Oligodendrocyte Glycoprotein

Myelin oligodendrocyte glycoprotein (MOG) is an integral membrane protein expressed in CNS oligodendrocytes and outermost myelin layers. Anti-MOG Abs cause myelin destruction (demyelination) in animal models of multiple sclerosis. A study indicates that MOG-specific IgGs are most frequently found in serum of patients with relapsing-remitting multiple sclerosis and only marginally in secondary progressive multiple sclerosis, but not at all in primary progressive multiple sclerosis (Lalive et al. 2006). Conclusions of this study are: (i) epitopes displayed on native, glycosylated MOG expressed in vivo are early targets for pathogenic Abs; (ii) these Abs, which are not detected in solid-phase assays, might be the ones to play a pathogenic role in early multiple sclerosis with predominant inflammatory activity; and (iii) the cell-based assay provides a practical serologic biomarker for early detection of CNS autoimmune demyelination including its preclinical stage.

Brain N-Acetylaspartylglutamate as Biomarker of Cognitive Function in MS

Half of all patients with multiple sclerosis experience cognitive impairment, for which there is no pharmacological treatment. Use of magnetic resonance spectroscopy (MRS), to examine metabolic changes in the hippocampi of multiple sclerosis patients and comparison of the findings to performance on a neurocognitive test battery has revealed that N-acetylaspartylglutamate (NAAG) concentration correlates with cognitive functioning (Rahn et al. 2012). Specifically, patients with cognitive impairment had low hippocampal NAAG levels, whereas those with normal cognition demonstrated higher levels. The authors of this study then evaluated glutamate carboxypeptidase II (GCPII) inhibitors, known to increase brain NAAG levels, on cognition in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis. Whereas GCPII inhibitor administration did not affect physical disabilities, it increased brain NAAG levels and dramatically improved learning and memory test performance compared with vehicle-treated EAE mice. These data suggest that NAAG is a biomarker for cognitive function in multiple sclerosis and that inhibition of GCPII could be used as a therapeutic strategy for prevention of cognitive decline or recovery of cognitive function.

Brain Imaging Biomarkers of Multiple Sclerosis

MRI Biomarkers of Multiple Sclerosis

MRI findings are important criteria for the diagnosis of multiple sclerosis and MRI has been used as a surrogate biomarker in clinical trials of new therapies. Earlier studies of serial MRI scans show that new inflammatory activity occurring at a rate seven to tenfold greater than the clinical events. Subsequent studies have convincingly demonstrated that the presence of active inflammation, as detected with Gd + lesions, is associated with a higher likelihood of relapse activity. Numbers and volumes of Gd + enhancing lesion are among the most robust MRI biomarkers of multiple sclerosis and short-term disability progression (Sormani et al. 2010). However, there is still lack of validated biomarkers of long term outcomes.

Although decrease in relapse rate or slowing of disability progression are now the primary outcome measures in phase III clinical trials, changes in lesion burden detected by MRI remain important secondary outcome measures. New therapies developed to slow neurodegeneration or promote remyelination in multiple sclerosis will require the use of advanced multimodal MRI techniques that capture structural as well as functional changes, and adoption of these biomarkers into multicenter trials will be technically challenging (Sicotte 2011).

Although classically MS lesions are in the white matter of the CNS, recent pathological and imaging studies have shown that cortical demyelination is common in MS and more extensive than considered previously (Popescu and Lucchinetti 2012). Three types of cortical lesion described in the cerebral and cerebellar cortices of MS patients are subpial, intracortical and leukocortical lesions are the MS. Cortical demyelination may be the biomarker of progression of MS with irreversible disability, epilepsy and cognitive impairment. Therefore, grey matter damage, global as well as regional, has the potential to become a biomarker of disease activity, complementary to the currently used MRI biomarkers (global brain atrophy and T2 hyperintense lesions). Furthermore, it may improve the prediction of the future disease course and response to therapy in individual patients and may also become a reliable additional surrogate biomarker of treatment effect (Horakova et al. 2012).

Molecular Imaging

PET imaging with the following tracers can assess inflammation, demyelination, neuronal damage, and astrocyte activation (Moccia and Ciccarelli 2017):

- TSPO(^{11}C-PK11195) targets inflammation andaffects chronic white matter lesions, gray matter volume as well as hippocampal volume.
- ^{18}F-FDG targets inflammation and affects white matter lesions.
- ^{11}C-flumazenil targets neuronal damage and affects gray matter volume.
- 11C-acetate targets astrocyte activation and affects gray matter volume.

In future clinical trials, evaluation of remyelination will require a reliable and quantifiable myelin biomarker to be used as a surrogate biomarker. Currently used MRI assessment lacks specificity for evaluating the level of remyelination within the brain. A synthesized fluorescent molecule, 1,4-bis(p-aminostyryl)-2-methoxy benzene (BMB) binds selectively to myelin both ex vivo and in vivo. The binding of BMB to myelin allows the detection of demyelinating lesions in an experimental autoimmune encephalitis model of demyelination and enables quantification of myelin loss in dysmyelinating mutants. In MS brain, different levels of BMB binding differentiated remyelination in shadow plaques from either demyelinated lesions or normal-appearing white matter. After systemic injection, BMB crosses the BBB and binds to myelin in a dose-dependent and reversible manner. Finally, there is evidence that BMB, radiolabeled with ^{11}C, can be used in vivo to image CNS myelin by PET in baboon. These results provide a perspective for developing a brain myelin imaging technique by PET.

Biomarkers of Response to Therapy of Multiple Sclerosis

Future approaches to multiple sclerosis should integrate clinical and imaging data with pharmacogenomic and pharmacogenetic databases to develop prognostic profiles of patients, which can be used to select therapy based on genetic biomarkers.

DNA Motifs in the Blood as Biomarkers of Response to Treatment

Mass sequencing and assembly technologies have enabled the identification and quantification of unique cell-free DNA motifs in the blood of patients with multiple sclerosis (MS). The most common MS clinical syndrome, relapsing-remitting MS (RRMS), is accompanied by a unique fingerprint of both inter- and intragenic cell-free circulating nucleic acids as specific DNA sequences that provide significant clinical sensitivity and specificity (Beck et al. 2010). Coding genes that are differentially represented in MS serum encode cytoskeletal proteins, brain-expressed regulators of growth, and receptors involved in nervous system signal transduction. Although coding genes distinguish RRMS and its clinical activity, several repeat sequences, such as the L1 M family of LINE elements, are consistently different in all MS patients and clinical status versus the normal database. These data demonstrate that DNA motifs observed in serum are characteristic of RRMS and disease activity and are promising as a clinical tool in monitoring patient responses to treatment modalities.

Gene Expression

Gene expression profiling can characterize and quantitatively measure the expression profiles of genes selected based on their role in inflammation and their susceptibility to regulation by current multiple sclerosis treatment agents, interferon-β

(IFN-β) and glatiramer acetate. IFN-inducible genes are universally upregulated after in vitro treatment with IFN-β while the expression of other selected genes encoding cytokines and molecules related to T cell trafficking, activation and apoptosis is variably affected. IFN-β and glatiramer exhibit distinctive and characteristic regulatory effects on the expression of the selected genes. The technology serves as the basis for simple and sensitive assays for detection of IFN-β neutralizing antibody based on the blocking effect of serum antibodies on the known regulatory properties of IFN-β. This provides important information on the immunoregulatory properties of IFN-β and glatiramer and supports potential clinical applications of this technology in detection of neutralizing antibody and evaluation of treatment responses in multiple sclerosis patients.

Expression profiling is being pursued further to study RNA-based biomarkers for multiple sclerosis in the broader context of each patient's genetics, protein markers, family history, and clinical information. The aim is to find diagnostic biomarkers for active or stable disease, and response biomarkers for currently available therapies.

Expression of genes as biomarkers of response to IFN-β therapy in multiple sclerosis is shown in Table 14.9. Biomarkers will enable responders and nonresponders to drugs to be identified, increase the efficacy and compliance, and improve the pharmaco-economic profile of these drugs. Systems biology can be used to integrate biological and clinical data for developing personalized treatment of multiple sclerosis (Martinez-Forero et al. 2008).

Lymphocyte Subsets as Biomarkers of Therapeutic Response

Lymphocyte subpopulations (LS) have been investigated in RMS patients during 1 year of treatment with fingolimod and simple as well as multivariate logistic regression models were performed (Paolicelli et al. 2017). ROC (receiver operating curve) analysis identified cut-off values of LS predicting a higher risk of relapses and of Gd + lesions. A fingolimod-induced re-modulation of the immune system was demonstrated suggesting that LS can predict clinical response to fingolimod treament in RMS.

Neurofilaments

Neurofilament protein subunits are potential CSF biomarkers for disease progression in MS. Neurofilament light (NfL) subunit can reflect acute axonal damage mediated by inflammatory mechanisms and can imply prognostic value for conversion from clinically isolated syndrome (CIS) to definite MS (Teunissen and Khalil 2012). The neurofilament heavy subunit may rather reflect chronic irreversible damage and has prognostic value for disease progression or disability. NfL is a biomarker of therapeutic response in MS as it is reduced by treatment with natalizumab, mitoxantrone/rituximab, and fingolimod. The neurofilament intermediate subunit has not yet been studied.

Table 14.9 Gene expression as biomarker of response to interferon-β in multiple sclerosis

Gene	Protein	Function	Comments
CASP3	Caspase 3	Regulation of apoptosis	Expression predicts response to IFN-β
CAST	Calpastatin	Cell adhesion	Response to IFN-β correlates with gene polymorphisms
COL25	Collagen type XXV	Extracellular proteoglycans	Response to IFN-β correlates with gene polymorphisms
FLIP	FLIP	Regulation of apoptosis	Gene expression predicts response to IFN-β
GPC5	Glypican 5	Ion channel regulation	Response to IFN-β correlates with gene polymorphisms
HAPLN1	Hyaluronan proteoglycan link protein	Extracellular proteoglycans	Response to IFN-β correlates with gene polymorphisms
IAP-1, IAP-2	IAP, IAP2	Inhibit caspase activation and apoptosis	IFN-β reduces gene expression in B lymphocytes
MMP-9	Matrix metalloproteinase-9	Disruption of BBB, immune cell migration into the CNS, and myelin degradation	IFN-β suppresses MMP-9 mRNA
MX1	Myxovirus resistance protein 1	IFN-β-induced protein with an antiviral effect	MX1 is a biomarker of the response to IFN-β
STAT	JAK family of proteins	Regulation of expression of genes that mediate biological effects of INF-β	IFN-β activates JAK-STAT signaling pathway
TRAIL	TNF-related apoptosis-inducing ligand	Regulation of apoptosis	TRAIL expression is a marker of IFN-β clinical efficacy

Studies showing higher NfLor heavy subunit levels to be altered upon treatment regimes indicate their potential clinical value in monitoring treatment or side effects. Future studies should be aimed at the optimization, standardization and interlaboratory implementation of the assays and address the predictive value of these biomarkers.

Vitamin D as Predictor of Activity and Progression of MS

The Betaferon/Betaseron in Newly Emerging multiple sclerosis For Initial Treatment study was a randomized trial originally designed to evaluate the impact of early vs delayed interferon β-1b treatment in patients with clinically isolated syndrome.

Serum 25(OH)D concentrations were measured at baseline and 6, 12, and 24 months. Results showed that in treated patients, low 25(OH)D levels early in the disease course are a strong risk factor for long-term MS activity and progression (Ascherio et al. 2014). The role for vitamin D in MS requires further investigation.

CSF Biomarkers in Multiple Sclerosis

CSF Cystatin C as a Biomarker of Multiple Sclerosis

Analysis of CSF samples from patients with multiple sclerosis by SELDI-TOF MS has revealed a unique protein of 12.5 kDa that is 100% specific for MS. Low levels of this protein were also found in some patients with HIV infection. Tandem mass spectroscopy of a tryptic digest of this 12.5 kDa protein identified it as a breakdown product of full-length cystatin C (13.4 kDa), an important inhibitor of cysteine proteases including the cathepsins. Although total cystatin C levels in the MS patients was not different compared with controls, the patients with the highest 12.5/13.4 peak ratios also had the greatest cathepsin B inhibitory activity. The biomarker 12.5 kDa cystatin might be useful in identifying a subgroup of patients with MS or identifying those at risk for this disorder. It can also be used to monitor treatment by measuring its levels in CSF.

Detecting Autoantibodies in Multiple Sclerosis

The identification of autoantibodies (autoAbs) in multiple sclerosis as specific biomarkers has became a relevant target. An aberrant N-glucosylation is a fundamental determinant of autoAb recognition in multiple sclerosis and CSF114(Glc), an antigenic probe accurately measuring IgM autoAbs in the sera of a multiple sclerosis patient, has been developed as disease biomarker. The relevance of CSF114(Glc) is demonstrated by its clinical application and correlation with disease activity and prognosis. CSF114(Glc), a structure-based designed glycopeptide, is able to recognize, by ELISA, the presence of specific IgM autoAbs in the sera of a multiple sclerosis patient population but not in blood donors and other autoimmune conditions. AutoAbs specific for CSF114(Glc) isolated from multiple sclerosis patients recognized myelin and oligodendrocyte antigens by immunohistochemistry but not other nonrelevant tissues. CSF114(Glc) is demonstrated as a reliable, specific probe in a longitudinal study of untreated multiple sclerosis patients. Development of IgG/IgM anti-CSF114(Glc) Abs paralleled clinical activity and brain lesions shown on MRI. Therefore, a CSF114(Glc)-based immunoassay on sera may have important prognostic value in monitoring multiple sclerosis disease progression and for optimal guidance of therapy.

Switch-Associated Protein 70 Antibodies in Multiple Sclerosis

Switch-associated protein 70 (SWAP-70) antibody is involved in several pathogenic mechanisms including germinal center formation, isotype switching and adhesion of inflammatory cells to vascular endothelial cells, all of which are crucial factors in MS pathogenesis of multiple sclerosis. A study has investigated possible correlation among levels of SWAP-70 antibody, measured humoral factors and disability scores in to find a biomarker that can be used as a predictor of relapsing remitting multiple sclerosis (Türkoğlu et al. 2014). ELISA studies showed high-titer SWAP-70 antibodies in 61.5% of sera obtained during the attack period and 34.6% of sera obtained during remission. There was a significant inverse correlation between SWAP-70 antibody levels and expanded disability status scale scores, CXCL10, soluble VCAM-1, CXCL13 and soluble VLA-4 levels. These results showed that SWAP-70 antibodies could be potentially utilized as relapse and prognostic biomarkers in MS. Whether or not SWAP-70 antibodies have any effect on disease mechanisms requires further investigation.

Gelsolin as a Biomarker of Multiple Sclerosis

Gelsolin, first discovered as an intracellular actin-binding protein involved in cell, has been recently implicated in a number of diseases. Plasma gelsolin is an abundant secretory protein that circulates in extracellular fluids of humans. While the true function of plasma gelsolin is not known, clinical and animal studies have shown that depletion of plasma gelsolin by injury and inflammation is associated with adverse outcomes. The proposed mechanism of gelsolin depletion is that it binds abundant actin in cells exposed by tissue breakdown. More recently, gelsolin was found to bind bioactive inflammatory mediators, lysophosphatidic acid, diadenosine phosphate, Aβ peptide, platelet-activating factor and possibly others. Evidence for gelsolin as a biomarker for multiple sclerosis is based on the following observations:

- Plasma gelsolin levels are reduced in an animal model of multiple sclerosis.
- The reduction in the plasma gelsolin levels precedes the manifestations of MS.
- Gelsolin administration prevents and/or suppresses the manifestation of the disease.

Matrix Metalloproteinases as Biomarkers in Multiple Sclerosis

Matrix metalloproteinases (MMPs) have been implicated in the pathogenesis of multiple sclerosis. Their suggested role includes the disruption of the BBB, immune cell transmigration into the CNS, and myelin degradation. A polymorphism in the

microsatellite of the promoter region of MMP-9, modulating its expression, could play a role in susceptibility to multiple sclerosis. Interferon-β treatment is associated with significant suppression of MMP-9 and MMP-7 mRNA in relapsing-remitting multiple sclerosis patients. Divergent expression of MMP-2 between relapsing-remitting and secondary-progressive patients compared with controls has been observed supporting the evidence for distinct immune mechanisms in two forms of the disease. MMPs may be considered as potential biomarkers for response to treatment as well as targets for immunotherapy in multiple sclerosis.

Oligoclonal Bands as Biomarkers of MS

Oligoclonal bands (OCBs) are bands of immunoglobulins that are seen when a patient's blood serum, or CSF is analyzed. A study was done to predict conversion from clinically isolated syndrome to clinically definite MS (CDMS) in a large international cohort. Results showed validation of MRI lesion load, and OCB as the strongest independent predictors of conversion to CDMS in this multicenter setting (Kuhle et al. 2015). OCB positivity was associated with higher Epstein-Barr nuclear antigen 1 IgG titres. OCB positive MS patients have twice the rate of conversion to RRMS.

Serum Proteomic Pattern Analysis in Multiple Sclerosis

Sera from relapsing-remitting multiple sclerosis patients and healthy controls have been tested with MALDI-TOF-MS and analysis of data, using proteomic spectral pattern recognition software, reveals distinct proteomic patterns in the disease group. MALDI-TOF/MS, in combination with serum proteomic pattern analysis, could be useful in the diagnosis, and a larger, masked trial to identify proteomic spectral patterns characteristic of relapsing-remitting, primary progressive and secondary progressive variants of MS is justified.

T Cells as Biomarkers of Multiple Sclerosis

T cells are principal components of many autoimmune diseases acting either as primary pathogens or helping B cells to produce antibodies, and perform both functions such as in multiple sclerosis. Because of the link between T cells and onset as well as progression of multiple sclerosis, several studies have compared biomarkers on the surface of T cells and disease status. CD45RA is a marker for naive T lymphocytes and intercellular adhesion molecule-3 (ICAM-3) is expressed on resting lymphocytes. CD45RA + ICAM-3 + lymphocyte ratio in peripheral blood might

indicate immunosenescence in multiple sclerosis but it is not certain whether it is also influenced by IFN-β 1b treatment.

Analysis of lymphocyte subsets and humoral immune parameters in peripheral blood and CSF samples to determine the immunological effects of high-dose intravenous methylprednisolone reveals that early clinical improvement is significantly associated with a decrease in CSF CD4(+)CD29(+) helper inducer T cells, whereas they are nearly unchanged in patients who show no improvement.

Concluding Remarks and Future Perspective for Biomarkers of Multiple Sclerosis

Currently, most of the efforts for biomarker development in multiple sclerosis has been in the area of monitoring disease activity. MRI and serum biomarkers that help monitor therapeutic efficacy such as the titer of antibody to β-interferon are established in clinical practice. As the armamentarium of available therapies for multiple sclerosis broadens, little is known about factors that predict treatment response in individual patients to a specific drug. Analogous to cancer therapy, the successful therapeutic strategy in multiple sclerosis might ultimately involve the combination of different therapeutics targeting several dominant pathophysiological processes. The development of these process-specific and personalized therapies will be impossible without the use of biomarkers that reflect the targeted process, can select patient population in which the targeted process is prevailing and can aid during the more rapid screening of therapeutic agents in the early phase of their development. According to the Multiple Sclerosis Research Center of New York, a disease 'activation' panel of CSF biomarkers should include the following: IL-6 or its soluble receptor, nitric oxide and nitric oxide synthase, osteopontin, and fetuin-A. Ongoing work with biomarkers that reflect drug bioavailability and factors that distinguish between medication responders and nonresponders are also under investigation (Harris and Sadiq 2009).

Although a number of studies have been performed to identify biomarkers of multiple sclerosis, most of them have some flaws either in terms of selection of patient populations or in terms of study design and methodological issues. The following suggestions have been made to define guidelines to be followed in future multicenter studies:

- Strict selection and stratification of multiple sclerosis patients according to well defined criteria, with a special preference for newly diagnosed untreated patients.
- Size of sample population should be large.
- The methods used in immuno and MRI monitoring should be standardized with particular focus on data reproducibility.
- Long-term follow-up of serially measured clinical, immunological and radiological biomarkers.
- Design of independent studies for the validation of new candidate biomarkers.

Biomarkers of Cerebrovascular Disorders

Several neurological disorders due to disturbances of cerebral blood flow or diseases of vessels involved in blood supply of the brain. The most common of these is stroke.

Biomarkers of Stroke

Stroke may be hemorrhagic (rupture of an artery) or thrombotic (blockage of an artery); 88% of all strokes are due to cerebral ischemia. The classic definition of ischemic stroke is a sudden, focal neurologic deficit lasting >24 h, confined to an area of the brain or eye perfused by a specific artery, and presumed to be of vascular origin. Each year, >5 million people worldwide die from stroke. Stroke is the third leading cause of death in the US and the third leading cause of serious long-term disability; >700,000 persons experienced a stroke every year, and 160,000 of these die.

The TOAST (Trial of Org 10,172 in Acute Stroke Treatment) classification divides ischaemic strokes into the following subtypes:

- Ischemic stroke due to large artery atherosclerosisIschemic
- Ischemic stroke due to cardioembolismIschemic
- Ischemic stroke due to small vessel occlusionIschemic
- Ischemic stroke of undetermined etiologyIschemic
- Ischemic stroke of other determined etiology

Because of the etiological heterogeneity and the fact that prognosis, risk of recurrence and management options differ greatly between subtypes, it is important to identify rapidly and accurately the underlying pathological mechanism leading to ischemic stroke. In acute stroke, a blood biomarker can be any quantifiable entity that reflects the manifestation of a stroke-related process. The most useful application of stroke biomarkers is in areas where information from traditional clinical sources is limited.

An ischemic stroke is diagnosed when typical clinical features are present without obvious evidence of hemorrhage on neuroimaging. Special diagnostic procedures include brain imaging with CT or MRI; and vascular imaging with CT angiography, Doppler, or magnetic resonance angiography. Routine hematology and biochemical studies are done but no special studies are usually done for biomarkers. Currently the key diagnostic questions considered for the biomarkers are:

- Is this a stroke?
- Is it an ischemic or a hemorrhagic stroke?
- If ischemic stroke, is it transient ischemic attack (TIA) or cerebral infarction?
- If cerebral infarction, is the patient likely to develop cerebral edema?
- If thrombolytics treatment is considered, is the patient likely to develop hemorrhage?

Various biomarkers of stroke are listed in Table 14.10. Some of these will be described in the following text.

Table 14.10 Biomarkers of stroke

Antibodies
Antiphospholipid antibodies
Phosphatidylethanolamine antibodies
B-type neurotrophic growth factor
Biomarkers of blood-brain barrier disruption in stroke
Matrix metalloproteinase-9 (MMP-9)
Biomarkers of brain damage and degeneration
C-tau
Biomarkers of excitotoxicity
NMDA receptor subtype NR2A
Biomarkers of oxidative stress
Biomarkers of inflammation
C-reactive protein (CRP)
Interleukins: IL-1 and IL-6
Intercellular adhesion molecule 1
Lipoprotein-associated phospholipase A2 (Lp-PLA2)
Matrix metalloproteinase-9
Serum amyloid A
Tumor necrosis factor α
Biomarkers of cardioembolic stroke
Plasma brain natriuretic peptide
Biomarkers of cerebral vasospasm
Angiopoietin-1 (Ang-1)
Gene expression after cerebral ischemia
Glial biomarkers of stroke
S100b
Glial fibrillary acidic protein
Homocysteine
Neurochemical biomarkers of cerebral ischemia
Lactate concentration in the brain increased
N-acetyl-aspartate in the brain decreased
Nucleosomes
Nucleoside diphosphate kinase A
Miscellaneous protein biomarkers
Glutathione S-Transferase-π
Heat shock proteins
Monocyte chemotactic protein-1
Neuron-specific enolase
Park 7 protein encoded by PARK7 gene
Visinen-like protein 1 (VLP-1)

Most of the listed biomarkers have little practical use in stroke. Elevated level of homocysteine a sulfur-containing amino acid derived from metabolism of methionine, is a risk factor for stroke. Antiphospholipid antibodies are biomarkers of an increased risk of stroke, particularly in individuals <50 years of age.

Etiological Biomarkers of Ischemic Stroke

Etiological blood biomarkers are associated with pathophysiological processes in the human body leading to an ischemic stroke. Ideally, they should be reliable and simple to measure; they should help scientists to gather further meaningful information about the etiology of ischemic stroke. Specifically, they should improve the identification of patients who need faster initiation of specific treatments, ultimately leading to a better outcome. PubMed search until 2014 identified the role of 22 selected blood biomarkers in the context of stroke etiology shown in Table 14.11 (Sonderer and Katan 2015).

To specifically address the question on etiological biomarkes in acute stroke, the BIOmarker SIGNAture of Stroke AetioLogy Study (BIOSIGNAL) – a multicenter international study funded by the Swiss National Science Foundation – was designed and has begun enrolling patients across Europe.

Brain Natriuretic Peptide as a Biomarker for Cardioembolic Stroke

Plasma brain natriuretic peptide has been linearly associated with atrial fibrillation, heart failure, chronic renal failure, and left atrial diameter, independently. Furthermore, atrial fibrillation, mitral regurgitation, plasma brain natriuretic peptide, and left atrial diameter are statistically significant independent predictors of cardioembolic stroke in the multivariable setting. Therefore, cardioembolic stroke can be strongly predicted with atrial fibrillation and plasma brain natriuretic peptide, which can be a surrogate marker for cardioembolic stroke (Yukiiri et al. 2008).

Brain Lactate and N-Acetylaspartate as Biomarkers of Stroke

Proton magnetic resonance spectroscopic imaging (1 H-MRS) and and diffusion-weighted imaging have been used to measure the cerebral lactate and N-acetylaspartate (NAA) levels in acute cerebral ischemia (Muñoz Maniega et al. 2008). Lactate is significantly increased whereas NAA is significantly decreased. The progressive fall in NAA suggests that some additional neuronal death may continue beyond the first few hours for up to 2 weeks or longer. If this mechanism is confirmed, it may be possible that interventions to limit this ongoing subacute tissue damage might add to the benefit of hyperacute treatment of stroke, making further improvements in outcome possible.

Table 14.11 Etiological blood biomarkers of ischemic strokes due to large artery atherosclerosis

Biomarker	Description	References
Adiponectin	Adipocytokine of adipose tissue, reduction of atherosclerotic development, improve insulin resistance	Kuwashiro et al. (2014)
CD62P	P-selectin, stimulated by thrombin, recruitment of leucocytes in inflammation, binding of thrombocytes	Tsai et al. (2009)
CD63	Cell surface protein, involved in adhesion of blood cells to endothelial cells	Tsai et al. (2009)
C-reactive protein	Inflammatory biomarker	Zeng et al. (2013)
CXCL16	Scavenger receptor and chemokine, involved in oxLDL uptake in monocytes, enhances atheriosclerosis	Ma et al. (2014)
Decorin	Matrix proteoglycan, component of connective tissue, binds to type I collagen fibrils and is involved in matrix assembly (cell cycle)	Xu et al. (2012)
LIGHT	Tumour necrosis factor ligand, proinflammatory prothrombotic effects on tissue, induces atherosclerosis	Liu et al. (2008)
LP PLA2	Platelet activating factor, increases inflammatory reaction	Katan et al. (2014) Delgado et al. (2012)
sCD40L	Binds CD40 on APCs, promotes inflammatory action on endothelial cells	Oberheiden et al. (2012) Wang et al. (2013)
Serum lipoprotein A	Lipoprotein, involved in atherosclerosis	Kim et al. (2010)
Total serum cholesterol	Hydrophobic sterol molecule, found in cell membranes, induces atherosclerosis?	Cui et al. (2012)

APC antigen presenting cell, *CD* cluster of differentiation, *CXCL16* C-C-C motif chemokinreceptor 16, *LIGHT* lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T lymphocytes, *LP PLA2* lipoprotein-associated phospholipase A2, *oxLDL* oxidized low density lipoprotein, *TIA* transient ischemic attack

Monitoring the level of lactate by 1 H-MRS has been used to evaluate the effect of hyperbaric oxygen (HBO) in a progressive ischemic stroke patient (Lee et al. 2008). Elevated lactate levels returned to normal within 11 days under HBO, whereas constantly high levels usually persist for more than 1 month after ischemia. Brain lactate level can be used as a biomarker of cerebral ischemia and for evaluation of treatment.

CRP as Biomarker of Risk of Stroke

Elevated levels of CRP increase the risk of stroke. Levels of inflammatory biomarkers associated with atherosclerosis, including high sensitivity CRP (hsCRP), are elevated and appear to be stable for at least 28 days after first ischemic stroke.

Admission CRP is associated with stroke severity and long-term mortality when measured at least 24 h after onset. The Bergen stroke study showed a crude association between high CRP and short-term functional outcome, which is likely secondary to stroke severity (Idicula et al. 2009). Thus CRP is an independent predictor of long-term mortality after ischemic stroke.

Statin drugs substantially reduce CRP levels (see Chap. 15). Role of rosuvastatin in the prevention of stroke among men and women with elevated levels of CRP was tested in JUPITER (Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin) clinial trial (Everett et al. 2010). Rosuvastatin reduces by more than half the incidence of ischemic stroke among men and women with low levels of LDL cholesterol levels who are at risk because of elevated levels of hsCRP. This reduction in risk was almost entirely due to strokes caused by blood clots or reduced blood flow to the brain. No increase or decrease in risk of rare hemorrhagic strokes due to bleeding were observed during the trial.

The results are relevant for patient care and the prevention of stroke. Physicians can no longer assume that patients are at low risk for stroke simply because they have low LDL cholesterol. This trial has confirmed that patients with increased hsCRP are at increased stroke risk even if cholesterol levels are low, and provides supporting evidence for the efficacy of rosuvastatin in reducing risk of stroke.

CSF Biomarkers in Acute Stroke

The first immune response of the brain glial tissue to acute ischemia is the activation of CD4 cells to produce γ-interferon, which stimulates astrocytes to express HLA-II class antigens and to produce IL-1β. The latter stimulates phagocyte activity in glial tissue and induces production of IL-6 and TNF-α, the cytokines of initial local inflammatory reactions that trigger the subsequent development of the pro- and anti-inflammatory cytokine cascade. Sixth hours after stroke onset, CSF cytokine levels are elevated in patients as compared to controls. CSF of severe stroke patients has increased IL-6 content and electron paramagnetic resonance (EPR) signals of nitric oxide (NO) as well as lipoperoxiradical compared to less severe strokes (Beridze et al. 2011). This study established a positive correlation between the initial IL-6 content and ischemic lesion size as well as with NIH Stroke Scale score on the seventh day. Initial IL-6 and NO in CSF are considered to be the most reliable prognostic biomarkers for functional outcome of stroke at 1 month.

Gene Expression in Blood Following Ischemic Stroke

Ischemic brain and peripheral white blood cells release cytokines, chemokines and other molecules that activate the peripheral white blood cells after stroke. To assess gene expression in these peripheral white blood cells, whole blood was examined using oligonucleotide microarrays in patients within 24 h after onset of ischemic stroke (Tang et al. 2006). Blood samples within 3 h of onset were drawn before

patients were treated either with tissue-type plasminogen activator (tPA) alone or with tPA plus Eptifibatide (the Combination approach to Lysis utilizing Eptifibatide And Recombinant tPA trial). Most genes induced in whole blood at 2–3 h were also induced at 5 and 24 h. Genes induced after stroke were expressed mainly by polymorphonuclear leukocytes and to a lesser degree by monocytes and included: matrix metalloproteinase 9, S100, arginase I, carbonic anhydrase IV, lymphocyte antigen 96, monocarboxylic acid transporter, erythroblastosis virus E26 oncogene homolog, cytoskeleton-associated protein 4, N-formylpeptide receptor, ribonuclease-2, N-acetylneuraminate pyruvate lyase, BCL6, and glycogen phosphorylase. These genes correctly classified 70% of patients within 3 h of stroke and 100% of patients at 24 h after stroke. These data provide insights into the inflammatory responses after stroke in humans, and should be helpful in diagnosis, understanding etiology and pathogenesis, and guiding acute treatment and development of new treatments for stroke.

Glutathione S-Transferase-π

Glutathione S-Transferase-π (GST-π) belongs to a family of multifunctional enzymes involved in the detoxification processes and therefore provides protection to cells exposed to oxidative stress or chemical reagents. GST-π presents a wide tissue expression pattern and has been reported to be present in the CNS, with a strong expression in different areas of the brain and also in brain capillaries. In a study tested that 29 molecules, GST-π concentration was the most significantly elevated biomarker in the blood of stroke patients (Turck et al. 2012). GST-π can accurately predict the time of stroke onset in over 50% of early stroke patients and this test could, therefore, complement current guidelines for tPA administration and potentially increase the number of patients accessing thrombolytic therapy. These results established GST-π as a reliable indicator of anoxic and/or cerebrovascular injury.

Intercellular Adhesion Molecule 1 as Biomarker of Ischemic Stroke

Adhesion molecules play important roles in the pathophysiology of ischemic stroke. Serum levels of soluble intercellular adhesion molecules 1 (sICAM-1on admission of stroke patients are significantly higher than those of healthy controls and are associated with neurological deterioration of stroke. The serum level of sICAM-1 can be considered as a biomarker of neurological deterioration of ischemic stroke. Because of the role of sICAM-1 in genesis of endothelian disfunction and tissue damage associated with ischemic stroke, it is a a therapeutic target for prevention of the progression neurological deficit (Maksimova et al. 2014).

Lp-PLA2 and CRP as Biomarkers for Stroke

Increased levels of the inflammatory biomarkers, lipoprotein-associated phospholipase A2 (Lp-PLA2), and high-sensitivity C-reactive protein (hs-CRP) have been shown to be associated with an increased risk for ischemic stroke. Value of biomarkers for predicting 5-year stroke risk was evaluated in a prospective case-cohort in apparently healthy, middle-aged men and women in the Atherosclerosis Risk in Communities (ARIC) study (Nambi et al. 2009). With use of traditional risk factors, 86% of participants were categorized as low risk (<2%); 11%, intermediate risk (2% to 5%); and 3%, high risk (>5%), whereas the addition of hs-CRP, Lp-PLA2, and their interaction to the model reclassified 4%, 39%, and 34% of the low-, intermediate- and high-risk categories, respectively. It was concluded that Lp-PLA2 and hs-CRP may be useful in individuals classified as intermediate risk for ischemic stroke by traditional risk factors.

Matrix Metalloproteinase-9

Matrix metalloproteinase-9 (MMP-9) levels on admission were significantly elevated compared to controls and dropped in the first 72 h after stroke onset, except in patients with stroke progression and larger infarcts in the subacute phase (Brouns et al. 2011). This study showed that that the extent of blood-brain barrier (BBB) breakdown in hyperacute stroke relates to initial stroke severity, stroke evolution and long-term outcome. CSF/serum albumin ratio and kinetics of MMP-9 in blood are biomarkers for BBB disruption after acute ischemic stroke The kinetics of MMP-9 confirm its pivotal role in secondary brain damage after ischemic stroke.

miRNAs as Biomarkers of Stroke

Identification of specific miRNAs associated with stroke has been a challenge due to complexity of its pathology. A study showed that circulating miRNA profiles reflect not only the temporal progression of stroke but also the specific causes (Sepramaniam et al. 2014). A panel of 32 miRNAs, which could differentiate causes of stroke during the acute phase, was identified and verified using a customized TaqMan Low Density Array. Five miRNAs – miR-125b-2*, −27a*, −422a, −488 and −-627 – were found to be consistently altered in acute stroke irrespective of age or severity or metabolic complications. These miRNAs biomarkers have potential as diagnostics for stroke. Findings of a clinical study suggest that miR-150 polymorphisms may contribute to the development of ischemic stroke and may be biomarkers with potential to predict the risk of ischemic stroke (Choi et al. 2016).

Neuroserpin Polymorphisms as a Biomarker of Stroke

Neuroserpin, primarily localized to CNS neurons, inhibits the adverse effects of tissue-type plasminogen activator (tPA) on the neurovascular unit and has neuroprotective effects in animal models of ischemic stroke. A population-based case-control study of stroke among women aged 15–49 identified 224 cases of first ischemic stroke (47.3% African-American) and 211 age-matched control subjects (43.1% African-American). Neuroserpin SNPs chosen through HapMap were genotyped in the study population and assessed for association with stroke (Cole et al. 2007). Of the five SNPs analyzed, the A allele of SNP rs6797312 located in intron 1 was associated with stroke in an age-adjusted dominant model among Caucasians but not African-Americans. This study provides the first evidence that neuroserpin is associated with early-onset ischemic stroke among Caucasian women.

NMDA Receptors as Biomarkers of Excitotoxicity in Stroke

NMDA receptor is central to physiological and pathological functioning of neurons. Expression of NMDA receptors subunit NR2A is up-regulated in cerebral cortex reperfusion following experimental middle cerebral artery occlusion indicating that NR2A autoantibodies are biochemical markers of the excitotoxicity in cerebral ischemia. NR2A autoantibodies are reported to be sensitive serologic biomarkers capable of detecting cerebral ischemic events and, for ruling out intracerebral hemorrhage in conjunction with neurologic observation and neuroimaging. Preoperative serum concentrations of NR2A antibodies, but not S100B or CRP, are considered to be predictive of severe neurological adverse events after cardiopulmonary bypass procedures. However, these findings have not been reproduced independently, and several clinical trials with NMDA receptor antagonists for stroke have failed.

A study has reported the rapid and extensive proteolytic processing of NR2A, together with the scaffolding protein postsynaptic density-95 (PSD-95), induced by excitotoxic stimulation of cortical neurons in vitro and by transient focal cerebral ischemia (Gascon et al. 2008). Processing of the C terminus of NR2A is irreversibly induced by brief agonist exposure of NR2B-containing receptors, and requires calcium influx and the activity of calpain, also responsible for PSD-95 cleavage. The outcome is a truncated NR2A subunit that is stable and capable to interact with NR1 at the surface of neurons, but lacking the structural domains required for association with scaffolding, downstream signaling and cytoskeletal proteins. Therefore, a rapid and significant uncoupling of synaptic NMDA receptors from downstream survival pathways is expected to occur during ischemia. This novel mechanism induced by excitotoxicity helps to explain the failure of most therapies based on NMDA receptor antagonists.

Nucleosomes as Biomarkers of Stroke

Nucleosomes are cell death products that are elevated in serum of patients with diseases that are associated with massive cell destruction. The kinetics of circulating nucleosomes after cerebral stroke and their correlation with the clinical status have been investigated (Geiger et al. 2006). ELISA was used to analyze nucleosomes in sera of patients with early stroke daily during the first week after onset. Patients with with slight functional impairment showed a continuous increase in nucleosomes until day 5 followed by a slow decline. In contrast, patients with severe functional impairment showed a steeper initial increase reaching a maximum already on day 3. Although nucleosomes are nonspecific cell death markers, their release into serum after cerebral stroke correlates with the gross functional status as well as with the infarction volume and can be considered as biochemical correlative to the severity of stroke.

PARK7 and Nucleoside Diphosphate Kinase a as Biomarkers of Stroke

PARK7/DJ-1 and nucleoside diphosphate kinase A (NDKA encoded by NM23 gene) are increased in human postmortem CSF, a model of global brain insult, suggesting that measurement in CSF and, more importantly, in plasma may be useful as a biomarker of stroke. ELISA was used to measure PARK7 and NDKA in plasma of stroke in three independent European and North American retrospective studies and control subjects. Increases in both biomarkers were highly significant, with sensitivities of 54–91% for PARK7 and 70–90% for NDKA and specificities of 80–97% for PARK7 and 90–97% for NDKA. The concentrations of both biomarkers increased within 3 h of stroke onset. It is concluded that both PARK7 and NDKA may be useful plasma biomarkers for the early diagnosis of stroke. In addition, this study demonstrated the utility of analysis of postmortem CSF proteins as a first step in the discovery of plasma markers of ischemic brain injury. Genetic ablation of DJ-1 renders the brain more susceptible to cell death following ischemia-reperfusion in a model of stroke. Extracellular DJ-1 has shown a neuroprotective effects on reperfusion injury in an in vitro model of ischemic stroke (Han et al. 2017).

Visinen-Like Protein 1

Visinen-like protein 1 (VLP-1) is increased in patients serum after ischemic stroke as shown by ELISA-based approach and transcript profiling. VLP-1 can be measured at concentrations <100 ng/L. An initial step in the optimization process is establishing the specificity of available antibodies, using Western blot and immunostaing or other suitable techniques. Although this biomarker is promising, characterizing the clinical usefulness of this biomarker will be an extensive process.

Biomarker Panels for Stroke

There is as yet no single validated biomarker of stroke. A study has reported that main independent predictors of stroke versus conditions mimicking it were caspase-3 > 1.96 ng/ml, D-dimer >0.27 μg/ml, chimerin <1.11 ng/ml, secretagogin <0.24 ng/ml and MMP-9 > 199 ng/ml (Montaner et al. 2011). The model's predictive probability of stroke when the six biomarkers are above/below these cut-off levels was 99.01%. The combination of caspase-3 and D-dimer in the model appears to be the most promising to achieve a rapid biochemical diagnosis of stroke. If replicated, this approach could be used as a tool for urgent referral of stroke patients to hospitals in which acute treatments are available.

The Triage® Stroke Panel, a biochemical multimarker assay, detects BNP, D-dimers, MMP-9, and S100B protein and promptly generates a multimarker index of these values, which has been licensed for diagnostic purposes as it might increase the validity of the clinical diagnosis to differentiate between stroke imitating diseases and true ischemic strokes. A study conducted in the setting of acute MRI-proven ischemic stroke, Triage® Stroke Panel was not found to be of diagnostic validity (Knauer et al. 2012). The authors do not recommend this assay as it might lead to a unjustified time delay.

Future Prospects for Biomarkers of Stroke

Future studies should integrate biomarkers with clinical information and imaging data to answer the questions raised at the start of this section. The advantages of a blood test that can be done within the first 3 h of onset of acute stroke are immense, as important decisions for treatment start in this phase. Thrombolytic treatment of stroke is carried out no later than 3 h following the onset of stroke. The patient is often in transport following the onset and the test should have the point-of-care feasibility. At that stage the provisional diagnosis stroke is usually made by paramedical personnel and a reliable biomarker would be helpful. Imaging studies and specialized treatments are conducted after the patient reaches the hospital. Base levels of biomarkers at initial studies can be compared with repeat tests during the course of treatment and in the follow-up phase.

There are no rational therapies for stroke. Biomarkers would help in drug discovery and development. The future of biomarker research for stroke is promising but many challenges have to be met. A number of studies have demonstrated that novel tools to assess simultaneously multiple biomarkers can provide unique insight such as details on specific molecular participants in cell death cascades, inflammation, or oxidative stress in stroke (Kochanek et al. 2008). However, a systematic review of 21 studies testing 58 single biomarkers and 7 panels of several biomarkers, found limitations in the design and reporting although all showed either a high sensitivity or specificity (Whiteley et al. 2008). Three specific questions need to be answered when assessing new biomarkers of stroke (Foerch et al. 2009):

1. Can biomarkers augment the clinical examination and powerful brain imaging tools to enhance the accuracy of the diagnostic process?
2. Can biomarkers be used to help triage patients for thrombolytic therapy?
3. Can biomarkers help predict patients who are most susceptible to malignant infarction?

Many encouraging molecular candidates have been found that appear to match the known cascades of neurovascular injury after stroke. Currently, none of these biomarkers are validated for use in clinical practice. Larger clinical trials are warranted to establish the sensitivity and specificity of biomarkers for routine use in management of stroke.

Biomarkers of Cerebral Vasospasm

The term "cerebral vasospasm (CVS)"means "narrowing" or a contracted state of the cerebral arteries in vivo. Vasospasm following subarachnoid hemorrhage (SAH) is an important cause of cerebral ischemia and is the most frequent serious complication in survivors of SAH. CVS is usually detected by Doppler sonography or cerebral angiography. A number of publications have described the role of biomarkers as a precursor to clinical deterioration after SAH. However, their role is unclear.

Angiopoietin-1 (Ang-1) and −2 (Ang-2) are key players in the regulation of endothelial homeostasis and vascular proliferation. Angiopoietins may play an important role in the pathophysiology of CVS. A prospective study has investigated patients with SAH and healthy controls by measuring Ang-1 and Ang-2 in serum samples using commercially available ELISA (Fischer et al. 2011). Transcranial Doppler sonography was performed to monitor the occurrence of CVS. Patients, who developed Doppler sonographic CVS, showed significantly lower levels of Ang-1 with a sustained decrease in contrast to patients without Doppler sonographic CVS, whose Ang-1 levels recovered in the later course of the disease. In patients developing cerebral ischemia attributable to vasospasm significantly lower Ang-1 levels have already been observed on the day of admission. Thus Ang-1, but not Ang-2, is significantly altered in patients suffering from SAH and especially in those experiencing CVS and cerebral ischemia. The loss of vascular integrity, regulated by Ang-1, might be in part responsible for the development of cerebral vasospasm and subsequent cerebral ischemia. Ang-1 may serve as a biomarker of CVS.

The post hoc analysis of biomarker data derived from the "Simvastatin in Aneurysmal Subarachnoid Hemorrhage (STASH)" study has shown that baseline CRP levels may have predictive value in identifying good grade SAH patients who are at greater risk of a clinical deterioration (Turner et al. 2015). A targeted prospective study is required to validate and quantify this observation.

Biomarkers of Intracerebral Hemorrhage

Astroglial proteins such as glial fibrillary acidic protein (GFAP) and S100B are promising candidates for a biomarker able to differentiate between ischemic stroke (IS) and intracerebral hemorrhage (ICH) in the acute phase of stroke. GFAP is brain-specific intermediate filament protein found in astrocytes. It is a candidate biomarker ICH in the acute phase. GFAP is detected in the serum in >80% of ICH patients, but only in 5% of those with ischemic stroke (IS). A cutoff point of GFAP serum concentration of 2.9 ng/L was found to provide a sensitivity of 79% and a specificity of 98% for the differentiation of ICH from IS. The reason for this is hypothesized as the acute disruption of astroglial cells and the BBB in ICH leading to rapid release of GFAP in serum, in comparison to a more delayed release in IS. Because GFAP is also detected in the serum of patients with high-grade gliomas due to GFAP overproduction and BBB alteration, the specificity of GFAP for detecting ICH in the acute phase of stroke is reduced (Jung et al. 2007).

Study of protein profiles of plasma samples obtained from stroke patients has revealed ApoC-I and APoC-III to be potential biomarkers. APoC-III has a sensitivity of 94% and a specificity of 87% for differentiating ICH from IS.

Copeptin, the C-terminal part of provasopressin, which is a biomarker of other diseases such as acute myocardial infarction, has been reported to be a prognostic biomarker in patients with ICH (Zweifel et al. 2010). If this finding can be confirmed in larger studies, copeptin might be an additional valuable tool for risk stratification and decision-making in the acute phase of ICH.

Increased serum TNF-α, one of the main proinflammatory cytokines regulating an inflammatory response, has been demonstrated to be associated with increased mortality rate of ICH (Fang et al. 2007) and recurrent stroke (Welsh et al. 2008). Polymorphisms in the regulatory region of promoter may result in different TNF-α concentration. The SNPs -308A, −1031C, −863A, and -857 T have been shown to be associated with increasing TNF-α expression. A study from Taiwan showed modest effect of TNF-α polymorphisms on ICH risks and size with significant gender differences (Chen et al. 2010). A further study examining the correlation between the SNPs and serum TNF-α concentration would be useful.

Biomarkers of Hypoxic Brain Damage

Outcome after cardiac arrest is mostly determined by the degree of hypoxic brain damage. Patients recovering from cardiopulmonary resuscitation are at great risk of subsequent death or severe neurological damage, including persistent vegetative state. The early definition of prognosis for these patients has ethical and economic implications. Somatosensory evoked potentials (SEPs) are also used in this situation

and their loss is indication of poor outcome. However in some cases, SEPs may be normal or showed only diminished amplitude but the biomarkers of protein destruction, serum NSE and S-100B are elevated. The variable values may reflect different patterns of neuropathological damage caused by diffuse hypoxia. Therefore, a multi-modal approach with a combination of clinical, biochemical, and electrophysiological investigations is recommended to predict neurological outcome after cerebral hypoxia reliably.

Serum NSE has a prognostic value in predicting outcomes in patients early after in-hospital cardiac arrest. Those who died or remained in persistent vegetative state had significantly higher serum NSE levels initially. Early determination of serum NSE levels is a valuable ancillary method for assessing outcome after in-hospital cardiac arrest.

Biomarkers of Ischemic Brain Damage

A population-based study shows that NT-proBNP levels are associated with volumetric and microstructural MRI markers of subclinical brain damage in community-dwelling middle-aged and elderly subjects without dementia or clinical diagnosis of heart disease (Zonneveld et al. 2017). Several hypotheses can explain the link between cardiac dysfunction and subclinical brain damage, eg, decreases in CBF can cause cerebral microvascular damage or dysfunction of the BBB. Inflammatory factors associated with cardiac stress could also harm BBB, leading to increased permeability and damage to the brain. Further research, including follow-up brain MRI studies and measurements of NT-proBNP, will be needed to clarify the relationship between cardiac dysfunction and subclinical brain disease. One cannot rule out that the observed subclinical brain damage led to increased levels of NT-proBNP, but animal studies indicate that it is more likely that cardiac dysfunction affects brain changes rather than vice versa.

D-Dimer as a Biomarker of Cerebral Venous Thrombosis

D-dimer has already been mentioned as a biomarker of venous thrombosis and pulmonary embolism. As a hemostatic marker, it has been considered useful in acute ischemic stroke and cerebral venous thrombosis (CVT). Patients with CVT present with a wide spectrum of signs and symptoms; most common are headaches, seizures, hemiparesis, and altered consciousness. Imaging as performed by venous CT-angiography or MR-angiography has only a 1 to 2 in 10 chance to detect CVT when typical symptoms are present and D-dimer measurements are of limited clinical value because of false positive and negative results (Tanislav et al. 2011).

Biomarkers of Traumatic Brain Injury

Traumatic brain injury (TBI) is a major national health problem. At present, the primary clinical methods for the evaluation of TBI are the Glasgow Coma Scale (GCS), pupil reactivity, and CT of the head. While these indices have proven useful for stratifying the magnitude and extent of brain damage, they have limited usefulness for predicting adverse secondary events or detecting subtle brain damage. There is no definitive diagnostic test for TBI to help physicians determine the seriousness of injury or the extent of cellular pathology.

There is need for discovery and validation of better biomarkers for TBI. Desirable attributes of an ideal TBI biomarker are:

- Should be ideally available in blood rather than CSF
- A simple method for measurement that can be used at point-of-care
- Should correlate with structural injury to the brain and the clinical outcome
- Should give information on mechanism of neuronal injury.
- Marker level should be elevated within 24 h after TBI.
- Should correlation with other TBI diagnostics such as MRI and CT
- Sensitivity to subclinical or mild TBI
- Usable for prediction of efficacy of therapy

Technologies for Identification of Biomarkers of TBI

For a comprehensive evaluation of TBI, multiple biomarkers need to be correlated. These include biomarkers in CSF and blood in addition to brain imaging, neurophysiological studies (EEG and evoked potentials) and tests of cognitive function. Since TBI is an evolving process monitoring may involve emphasis on certain tests that are practical at a particular stage of head injury.

Cerebral Microdialysis for Study of Biomarkers of TBI

Microdialysis was introduced as an intracerebral sampling method for clinical neurosurgery and has also been used as a research tool to measure the neurochemistry of acute human brain injury. Peripheral blood, CSF and cerebral microdialysis have been used for proteomic studies. TBI Biomarkers discovered by proteomics are complementary to those identified by traditional approaches and should be validated in preclinical or clinical samples. Clinical studies have provided ample evidence that intracerebral microdialysis monitoring is useful for the detection of overt adverse neurochemical conditions involving hypoxia/ischemia and TBI. There is some data strongly suggesting that microdialysis alterations precede the onset of secondary neurological deterioration following TBI. These promising investigations have relied on microdialysis biomarkers of disturbed glucose metabolism

(glucose, lactate, and pyruvate) and amino acids. Others have focused on trying to capture other important neurochemical events, such as excitotoxicity, cell membrane degradation, reactive oxygen species and nitric oxide formation, cellular edema, and BBB dysfunction. It remains one of very few methods for neurochemical measurements in the interstitial compartment of the human brain and will continue to be a valuable translational research tool for the future. The future success of microdialysis as a diagnostic tool in clinical neurosurgery depends heavily on the choice of biomarkers, their sensitivity, specificity, and predictive value for secondary neurochemical events, and the availability of practical bedside methods for chemical analysis of the individual biomarkers.

Intracerebral microdialysis catheters with a high molecular cutoff membrane have been used to harvest interstitial fluid for study of protein biomarkers. Monitoring of interstitial T-tau and Aβ42 by using microdialysis may be an important tool when evaluating the presence and role of axonal injury following TBI (Marklund et al. 2009). These are biomarkers for AD as well and support the hypothesis that TBI as an important environmental risk factor for the development of AD.

Proteomic Technologies for Biomarkers of TBI

Neuroproteomic technologies are uniquely suited for the discovery of otherwise unnoticed TBI biomarkers and are being used for mapping changes in proteins after injury. This will be very useful for developing diagnostic predictors after CNS injury and to identify new therapeutic targets. SDS-PAGE prior to in vitro proteolysis and capillary LC-MS is a promising strategy for the rapid discovery of putative protein biomarkers associated with TBI without a priori knowledge of the molecules involved. Large-scale neuroproteomic research in CNS injury is being applied for immediate biomarker discovery and the systems biology approach is used for understanding of how the brain responds to trauma. Eventually, the knowledge gained through neuroproteomics could lead to improvement in diagnostics and therapeutics of CNS injury.

After TBI, brain cells can deteriorate following more than one pathway, and many genes and proteins may be involved. Cell death following TBI often has the morphological appearance of apoptosis. Studies of apoptosis pose special challenges since there are multiple apoptotic pathways, and apoptosis is extremely sensitive to a number of variables including injury type and magnitude, cell type and stimulation/antagonism of specific receptors. The molecular events occurring after TBI are just beginning to be understood. Elevated neuronal calcium levels activate a number of calcium-dependent enzymes such as phospholipases, kinases, phosphatases, and proteases, all of which can modulate post-TBI cytoskeletal protein loss. Caspase-3 is a member of the caspase family of cysteine proteases. Activated caspase-3 has many cellular targets that, when severed and/or activated, produce the morphologic features of apoptosis. Calpains are calcium-activated, neutral cysteine proteases with relative selectivity for proteolysis of a subset of cellular pro-

teins. Calpain activation has been implicated in different models of apoptosis and in different cell types, including neurons. Understanding of the contributions of calpains and caspases to cell injury/death following TBI may have important diagnostic and therapeutic implications. In vivo studies have provided evidence of caspase-3 activation following TBI. αII-spectrin is a major substrate for both calpain and caspase-3 cysteine proteases and considerable laboratory data exists on the potential utility of αII-spectrin degradation as a biomarker for TBI, including the ability to detect differences in severity of injury based on αII-spectrin detection in spinal fluid after TBI.

Initial research focused on proteolytic processing of cytoskeletal proteins such as lower molecular weight neurofilament 68 protein (NF-68) highlights their potential to provide useful information on activity of specific proteases such as μ-calpain and m-calpain. Importantly, 2D GE studies suggested dephosphorylation of NF-68 may be associated with NF protein loss following TBI, a post translational modification that could have significance for biomarker development. This important biomarker could provide important information on the pathophysiology of both dendritic and axonal damage after TBI. Importantly, NF-68 has been used to quantify axonal injury in closed head injury models. Since diffuse axonal injury is currently considered one of the most common types of primary lesions in patients with severe closed TBI, a biomarker that provides information on axonal injury could potentially have clinical utility. Banyan Biomarkers Inc. is focusing on diagnostics for TBI and has 42 novel protein biomarkers in its pipeline that can provide important information on the severity of injury, the location of the injury in the brain and the biochemical mechanisms underlying this injury.

Single Molecule Array technology (SiMoA™, Quanterix) is used to develop tests based on protein biomarkers of TBI in blood. Sports-related concussion in professional ice hockey players, which is associated with acute axonal and astroglial injury, can be monitored using tau, S-100, calcium-binding protein B, and neuron-specific enolase concentrations in serum, which may be developed into clinical tools to guide sport physicians in the medical counseling of athletes in return-to-play decisions (Shahim et al. 2014).

Systemy Biology Approach for Discovery of Biomarkers of TBI

Emerging systems biology strategies have been applied to existing TBI datasets to identify new biomarkers by analyzing the complex interweaving molecular pathways and networks that mediate the secondary cellular response through computational models that integrate these diverse datasets (Feala et al. 2013). As an example, the authors applied network and pathway analysis to a manually compiled list of 32 protein biomarker candidates from the literature to recover known TBI-related mechanisms, and generate hypothetical new biomarker candidates. Systems biology approach has been used in a rodent model of concussive injury to understand the impact of traumatic brain injury on gene regulation in the brain (Meng et al. 2017). Results of this study show that concussive brain injury reprograms genes

which could lead to predisposition to neurological and psychiatric disorders, and that genomic patterns from peripheral leukocytes has the potential as biomarkers to predict pathogenesis of traumatic brain injury.

Biomarkers of TBI

Biomarkers of TBI are listed in Table 14.12 and discussed in the following text.

Aβ as a Biomarker of TBI

The role of Aβ neuropathology and its significant changes in biofluids after TBI is controversial. An ultrasensitive digital ELISA approach (Quanterix Corporation) was used to assess Aβ42 concentrations and time-course in CSF and in plasma of patients with severe TBI and their relationship to injury characteristics, neurological status and clinical outcome was investigated (Mondello et al. 2014). The authors found decreased CSF Aβ42 levels in TBI patients acutely after injury with lower levels in patients who died 6 months post-injury than in survivors. Conversely, plasma Aβ42 levels were significantly increased in TBI with lower levels in patients who survived. A trend analysis showed that both CSF and plasma Aβ42 levels

Table 14.12 Biomarkers of traumatic brain injury

CSF biomarkers
Interleukin-6 (IL-6)
Myelin basic protein
Nerve growth factor (NGF)
Neuron-specific enolase (NSE)
Tau levels
Serum/blood biomarkers
Calcium-binding protein B
Glial fibrillary acidic protein (GFAP)
Hyperphosphorylated axonal neurofilament protein in serum
microRNA in peripheral blood mononuclear cells
Neurofilament heavy chain
Neuron-specific enolase
S100B protein
Spectrin N-terminal fragment (SNTF)
Ubiquitin C-terminal hydrolase-L1 (UCH-L1)
Tau
Imaging
Diffusion tensor imaging (DTI)

strongly correlated with mortality. A positive correlation between changes in CSF Aβ42 concentrations and neurological status as assessed by Glasgow Coma Scale was identified. Results suggest that determination of Aβ42 may be valuable for determining prognosis in patients with severe TBI as well as for monitoring the response of the brain to injury.

Diffusion Tensor Imaging in TBI

Traumatic axonal injury (TAI) is a common mechanism of TBI that is not readily identified using conventional neuroimaging. Diffusion tensor imaging (DTI) can detect microstructural disruption in white matter of the brain. DTI-derived data can be analyzed using global methods and each of these methods produce qualitatively comparable results, which are useful in clinical research and eventually in clinical practice (Marquez de la Plata et al. 2011). DTI measures of microstructural integrity appear robust for detecting white matter injury.

Glial Fibrillary Acidic Protein as Biomarker of TBI

Glial fibrillary acidic protein (GFAP) is a specific blood biomarker of astroglial injury. GFAP is a monomeric intermediate filament protein, a major constituent of the astroglial cytoskeleton. Blood levels of GFAP are increased in TBI patients on admission and have been correlated with both initial Glasgow Coma Scale (GCS) scores and brain imaging findings. GFAP has performed consistently as a biomarker in detecting mild to moderate TBI and correlated with lesions seen on CT scan (Papa et al. 2016). A prospective study showed that the level of GFAP significantly correlates with the initial severity of TBI not only at admission but also during the first 2 days after the injury suggesting that it has diagnostic value also beyond the first 24 h of injury (Posti et al. 2016). Elevations of this biomarker after the first 24 h may serve as a sign of secondary insults, progressive damage, or initial injury severity in cases of delayed medical care.

Hyperphosphorylated Axonal Neurofilament Protein

Hyperphosphorylated axonal neurofilament protein (pNF-H) is likely to be released from damaged and diseased neurons in significant amounts. A sensitive NF-H ELISA is capable of detecting picogram quantities of pNF-H in experimental animals. This assay shows that soluble pNF-H immunoreactivity is readily detectable in the sera of rats following various types of experimental spinal cord injury (SCI) and TBI, but is undetectable in the sera of normal animals. After an initial peak, a lot more of pNF-H is then released in the 2 or 3 days following CNS injury. This provides a window of opportunity to develop therapeutics for preventing delayed CNS damage following injury. These findings suggest that serum levels of pNF-H

immunoreactivity may be used as a biomarker to conveniently monitor neuronal damage and degeneration in experimental and presumably clinical situations. The test would be helpful in emergency rooms or in combat situations if it could be developed into a simple handheld device that could confirm brain or spinal injury and to determine its severity. Further studies are under way to determine whether pNF-H is detectable in people who have had strokes or who suffer from ALS or Alzheimer's disease and other serious damage and disease states of the nervous system. Pharmaceutical researchers can already use the technique to monitor the effectiveness of experimental medicines in animal models of stroke and TBI.

IL-6 and Nerve Growth Factor as Biomarkers of TBI

Secondary brain damage after TBI involves neuroinflammatory mechanisms that are mainly dependent on the intracerebral production of cytokines. Interleukin-6 (IL-6) may have a role both in the pathogenesis of neuronal damage and in the recovery mechanisms of injured neurons through the modulation of nerve growth factor (NGF) biosynthesis. A prospective observational clinical study of children with TBI, IL-6 and NGF upregulation in the CSF after injury was associated with better neurologic outcomes (Chiaretti et al. 2008). Based on these findings, NGF expression is a useful biomarker of brain damage following severe TBI. Moreover, the early upregulation of both IL-6 and NGF, which correlates with a favorable neurologic outcome, may reflect an endogenous attempt at neuroprotection in response to the damaging biochemical and molecular cascades triggered by traumatic insult.

Myelin Basic Protein

MBP is localized in the myelin sheath and constitutes approximately one third of the total protein of myelin from the human brain. TBI-mediated axonal injury causes secondary structural damage to the adjacent myelin membrane, instigating MBP degradation, which can be detected in serum. MBP levels are also raised in CSF following TBI. MBP, however, is not a specific biomarker of TBI as it is found in other neurological disorders as well.

Neurofilament Heavy Chain

Neurofilament heavy chain (NF-H) is a neuronal protein detected in the peripheral circulating blood after injury and raised levels can reflect the extent of the damage caused by blast TBI (bTBI). Blood samples were obtained prior to injury and at 6, 24, 72 h, and 2 weeks post-injury from animals with different severities of bTBI; protein levels were determined using reverse phase protein microarray technology (Gyorgy et al. 2011). Serum NF-H levels increased in a unique, rapid manner,

peaking at 6 h post-injury only in animals exposed to severe blast with poor clinical and pathological outcomes. The study concluded that the sudden increase in serum NF-H levels following bTBI may be a useful indicator of injury severity. If additional studies verify these findings, the observed early peak of serum NF-H levels can be developed into a useful diagnostic tool for predicting the extent of damage following bTBI.

Serum S100β as Biomarker of TBI

S100β is a 21 kDa c Ca^{2+}-binding protein found mainly in the cytosol of astroglial cells. The intracellular functions of this protein are radically different from its extracellular effects. At the intracellular level, S100β regulates multiple functions, including protein phosphorylation and degradation, cell motility and form, cellular proliferation and differentiation, Ca2+ homeostasis, and receptor transcription and regulation. At the extracellular level, S100β functions as a neuromodulatory signal, exhibiting concentration-dependent characteristics. Under physiological conditions, it acts as a neurotrophic factor, nearing nanomolar. In the presence of astrocyte damage or necrosis, however, its concentration increases to micromolar or sub-micromolar due to the passive release of intracellular S100β, and its effects become neurotoxic, directly provoking neuronal apoptosis, or indirectly stimulating the astrocytic release of nitric oxide. Furthermore, CSF and serum S100B levels have been correlated with outcome of TBI. Serum S100β is a sensitive biomarker of brain injury and indicatese severity of the injury. S100β, eliminated by renal excretion, has a biological half-life of 30 min to 2 h. Its values are not affected by hemolysis. MRS is capable of measuring S100B protein. Currently, 2 tests provide measurements of S100β levels in serum: Elecsys®S100 test (Roche Diagnostics) and Liaison Sangtec®100 system (DiaSorin SpA). However, studies have shown that these assays do not concur when analyzing serum S100β levels, making their results incomparable, which complicates efforts to compare different TBI-related studies

Large extracranial injuries also elevate S100β levels but S100β has a high negative predictive power, and the finding of a normal S100β value shortly after trauma should exclude significant brain injury with a high accuracy. Of all the reported biomarkers, only S100β in has consistently demonstrated the ability to predict outcome in adults in severe TBI (Kövesdi et al. 2010). S100β protein is a promising biomarker for the diagnosis, monitoring, and prognosis of TBI (Egea-Guerrero et al. 2012).

SNTF as a Biomarker for Predicting Cognitive Decline after Mild TBI

Although mild traumatic brain injury (mTBI) or concussion is not typically associated with abnormalities on CT, it can often cause persistent cognitive dysfunction. Therefore, new prognostic biomarkers are needed for mTBI to identify patients at

risk of cognitive decline at an early and potentially treatable stage. A study has quantified plasma levels of the neurodegeneration biomarker calpain-cleaved αII-spectrin N-terminal fragment (SNTF) from CT-negative mTBI subjects as well as normal uninjured controls, which were compared with findings from diffusion tensor imaging (DTI) and long-term cognitive assessment (Siman et al. 2013). The blood test identified SNTF in some of the orthopedic injury patients as well, suggesting that these injuries could also lead to abnormalities in the brain, such as a concussion, that may have been overlooked with existing tests. The blood biomarker test given on the day of the mTBI showed 100% sensitivity for predicting concussions leading to persisting cognitive problems, and 75% specificity for correctly ruling out those without functionally harmful concussions. An elevation in plasma SNTF corresponded with significant differences in fractional anisotropy and the apparent diffusion coefficient in the corpus callosum and uncinate fasciculus measured by DTI. Furthermore, increased plasma SNTF on the day of injury correlated significantly with cognitive impairment that persisted for at least 3 months, both across all study participants and also among the mTBI cases by themselves, indicating the possibility of identifying undiagnosed cases of mTBI. These data suggest that the blood level of SNTF on the day of a CT-negative mTBI may identify a subset of patients at risk of white matter damage and persistent disability. If validated in larger studies, SNTF could be a prognostic and diagnostic biomarker for the assessment as well as treatment of mTBI. This is important for deciding on return of athletes to sports or soldiers to military duty following mTBI. Larger studies are planned to determine the best time after concussion to measure SNTF in the blood in order to predict persistent brain dysfunction, and to evaluate the blood test for identifying when repetitive concussions begin to cause brain damage. SNTF as a biomarker is consistent with earlier research showing that calcium is dumped into neurons following a TBI, and neurodegeneration driven by calcium overload.

Tau as Biomarker of TBI

Tau proteins are microtubular binding proteins localized in the axonal compartment of neurons. Brain injury releases cleaved Tau proteins into the extracellular space where they are transported to the CSF. Tau protein in the CSF has been measured by a sandwich ELISA method. CSF Tau levels are elevated in patients with TBI. Furthermore, the elevation of CSF Tau levels in a comatose head-injured patient with an unremarkable CT scan indicates that CSF Tau levels are a more sensitive measure of axonal damage than CT. The correlation between the patient's clinical condition and CSF Tau levels suggests that CSF Tau levels may be a good predictor of severity of head injury and possibly patient outcome after discharge.

In rats subjected to controlled cortical impact-induced mild, moderate or severe levels of TBI, significant severity-dependent increase in C-tau levels have been observed in the cortex as early as 6 h after injury. Cyclosporin-A (CsA) significantly attenuated the TBI-induced increase in hippocampal C-tau levels and neuroprotectant effect was confirmed utilizing histologic measures of TBI-induced tissue loss.

These results suggest that C-tau is a reliable, quantitative biomarker for evaluating TBI-induced neuronal injury and a potential biomarker of neuroprotectant drug efficacy in the rat TBI model. Serum data suggests that C-tau levels are dependent both on a compromised blood-brain barrier as well as release of TBI biomarkers from the brain, which has implications for the study of human serum TBI biomarkers.

A study on collegiate atheletes to determine how acute plasma tau relates to prolonged return to play (RTP) after sports-related concussion (SRC) included cognitive tests, postural stability, and symptom reported to a consulting physician (Gill et al. 2017). Results showed that elevated plasma tau concentration within 6 h following a SRC was related to having a prolonged RTP, suggesting the usefulness of tau levels as a biomarker to make the decision about a return to play.

Ubiquitin C-Terminal Hydrolase-L1

A case-control study has examined concentrations of a novel brain injury biomarker, Ubiquitin C-terminal Hydrolase-L1 (UCH-L1), in CSF and serum of severe TBI patients and their association with clinical characteristics and outcome (Mondello et al. 2012). Using sensitive UCH-L1 sandwich ELISA, comparison of serum and CSF levels of UCH-L1 in TBI patients versus controls, showed significant elevation of UCH-L1 in acute phase and over the 7 day study period. Serum and CSF UCH-L1 Receiver Operation Characteristics curves further confirmed strong specificity and selectivity for diagnosing severe TBI versus controls. Area under the curve (AUC) values in serum and CSF were statistically significant up to 24 h. Furthermore, UCH-L1 levels in CSF and serum appeared to distinguish severe TBI survivors versus non-survivors within the study, with non-survivors having significantly higher and more persistent levels of serum and CSF UCH-L1. Cumulative serum UCH-L1 level > 5.22 ng/ml predicted death. It was concluded that serum levels of UCH-L1 appear to have potential clinical utility in diagnosing TBI, including correlating to injury severity and survival outcome. A prospective study showed that the level of UCH-L1 significantly correlates with the initial severity of TBI not only at admission but also during the first 2 days after the injury suggesting that it has diagnostic value also beyond the first 24 h of injury (Posti et al. 2016). Elevations of this biomarker after the first 24 h may serve as a sign of secondary insults, progressive damage, or initial injury severity in cases of delayed medical care.

Biomarkers of Inflicted TBI in Infants

Inflicted traumatic brain injury (iTBI), popularly called “the shaken baby syndrome”, is the leading cause of death from TBI in infants. Infants with iTBI are often misdiagnosed because doctors rarely receive a history that an infant has been shaken, the patients are too young to talk, and the symptoms such as vomiting and fussiness are common in many childhood illnesses. Infants who are misdiagnosed

may be inadvertently returned to a violent caretaker and be re-injured, sometimes with fatal consequences. Serum S100B is increased in the majority of children with acute TBI including well-appearing children with iTBI in whom the diagnosis might otherwise have been missed. Elevated serum neuron-specific enolase (NSE) levels are correlated with brain cell damage and correlation with Glasgow Coma Scale in TBI (Guzel et al. 2008). Cytochrome c, a biomarker of apoptosis, is increased in CSF from infants with inflicted brain injury from child abuse. Elevated serum and/or CSF concentrations of NSE, S100B, and MBP are sensitive and specific biomarkers that can be used to screen infant who are at increased risk for iTBI and may benefit from additional evaluation with a CT scan of the brain.

Biomarkers of Concussion

No routine tests are currently available for objective diagnosis of mild traumatic brain injury (mTBI)/concussion. Previously reported biomarkers for mTBI represented proteins released from the damaged neurons or glia. However, the low levels of these proteins and/or the complexity of assays used for their detection limits the implementation of these biomarkers in routine practice. A study on patients sustaining a concussion within the past 24 h has identified 4 candidate biomarkers: copeptin, galectin 3, matrix metalloproteinase 9 (MMP9), and occludin (Shan et al. 2016). Alterations in the blood levels of this panel of 4 biomarkers discerns with high accuracy patients with isolated concussion from uninjured individuals within the first 8 h after accident, and can also aid in diagnosing concussion in the presence of orthopedic injury. The proteins are readily measurable with standard assays, but the authors plan to develop a microfluidic chip that can derive reliable readings within 2 h (well within the duration of many emergency room visits). The proteins are potential therapeutic targets as well. Some of these inflammatory proteins may affect the integrity of the BBB, e.g. patients who suffer TBI not only suffer from the physical damage of the blow to the head but also from the resulting inflammatory response, especially within the first 24 h. High levels of MMP9 degrade the tight junction proteins at the BBB and allow undesirable substances to access the brain, which may increases neuroinflammation. One can think about a treatment that blocks MMP9. The biomarker panel may therefore eventually help not only with diagnosis, but may also aid treatment during a critical window of time.

Clinical Applications of Biomarkers of TBI

Biomarkers of neuronal, glial, and axonal damage such as neuron-specific enolase, S100B, and myelin basic protein, respectively, are readily detectable in biological samples such as serum or CSF and are being studied in patients with TBI. In addition, a number of studies have demonstrated that novel tools to assess

simultaneously multiple biomarkers can provide unique insight such as details on specific molecular participants in cell death cascades, inflammation, or oxidative stress. Multifaceted cellular, biochemical, and molecular monitoring of proteins and lipids is desirable as an adjunct to guide therapy and improve outcome in TBI. Biomarkers are useful as diagnostic, prognostic, and monitoring adjuncts in neurointensive care (Kochanek et al. 2008).

TBI biomarkers may have clinical utility in stratifying injury severity level, predicting adverse secondary events or outcomes, and monitoring the effectiveness of therapeutic interventions. As a biomarker source, serum offers several advantages over CSF, including ease of accessibility and reduced risk to the patient. Screened pooled serum samples obtained from severe TBI patients and age-, sex- and race-matched volunteers have been studied for biomarkers (Hergenroeder et al. 2008). Labeling with mass-balanced isobaric tags (iTRAQ), and analysis by LC-MS/MS revealed 31 candidate biomarkers whose serum abundance was altered after TBI. Changes in two candidate biomarkers – CRP and retinol binding protein (RBP) – were robust indicators of injury even at very acute time points. Analysis of serum RBP4 levels at 24–36 h post-injury indicates it may predict subsequent increases in intracranial pressure (ICP) with a sensitivity of 86% and specificity of 88%. These results support the use of serum as a source for discovery of TBI biomarkers, and indicate that serum biomarkers may have utility for predicting secondary pathologies such as elevated ICP associated with TBI. Changes in the expression profile of biomarkers such as microRNA in peripheral blood mononuclear cells may reflect molecular alterations following TBI that contribute to the onset and progression of TBI phenotypes including chronic traumatic encephalopathy (Pasinetti et al. 2010).

Biomarkers of CNS Infections

Compared to other disorders of the CNS, much less work has been done on biomarkers of primary infections involving the CNS. Many systemic infections also affect the brain and biomarkers may indicate the damage to the brain. S-100beta and neuron-specific enolase (NSE) are frequently increased and associated with brain injury in patients with severe sepsis and septic shock; S-100beta levels more closely reflect severe encephalopathy and type of brain lesions than NSE. All microorganisms can infect brain. Examples are given of viral and bacterial affections of the brain.

Biomarkers of Bacterial Meningitis

The mechanisms of CNS involvement in bacterial meningitis is not well understood. Several studies have provided substantial evidence for the key role of nitric oxide (NO) and reactive oxygen species in the complex pathophysiology of bacterial meningitis. One study has investigated serum and CSF levels of NO, lipid

peroxide (LPO, mediator of oxidative stress), and S-100B protein (mediator of astrocytes activation and injury) in children with bacterial meningitis (Hamed et al. 2009). Positive correlation was found between NO index with CSF WBCs; CSF-LPO with CSF-protein; total thiol with LPO indices; S-100B and Pediatric Glasow Coma Scores; CSF-LPO with CSF-S-100B; and serum-total thiol with serum S-100B. This study suggests that S-100B may be a biomarker of the severity and neurological complications of bacterial meningitis.

Increased levels of CSF 14-3-3 proteins have been reported in acute bacterial meningitis and they decrease after antimicrobial therapy. Therefore, CSF 14-3-3 protein levels should predict treatment outcomes. Serial 14-3-3 protein gamma isoform actually meets the major requirements for outcome prediction in the treatment of acute bacterial meningitis patients (Lu et al. 2008). Assay of the 14-3-3 protein gamma isoform can be added as a neuropathologic biomarker to the panel of conventional CSF parameters in meningitis.

An increase in adenosine deaminase (ADA) levels – an enzyme that plays a role in maturation of monocytes, macrophages and T lymphocytes – is observed in tuberculosis as well as other bacterial infections in which the cellular immunity response is actively involved (Karsen et al. 2011). The sensitivity and specificity for CSF ADA activity are markedly high in differential diagnosis of TB from non-TB. Hence CSF ADA activity may be used as a simple, cost-effective and reliable biomarker test for early differential diagnosis of TB.

Biomarkers of Viral Infections of CNS

Biomarkers of CNS HIV Infection

Although it is well recognized that HIV-1 can cause CNS dysfunction, current approaches to classification and diagnosis of this dysfunction rely on definitions of syndromes or measures of abnormality on neuropsychological testing. The interpretations of these definitions vary considerably, offer only limited sensitivity or specificity, and do not easily distinguish active from static brain injury. Replacing or supplementing these approaches with objective biologic measurements related to underlying disease processes would significantly advance classification, diagnosis, epidemiology, and treatment assessment. Two major approaches are being actively pursued with this aim: (1) analysis of soluble molecular biomarkers in CSF and blood; and (2) neuroimaging biomarkers using anatomic, metabolic, and functional measurements (Price et al. 2007).

Using a simple model of pathogenesis, an approach has been proposed for characterizing patients, selecting treatment targets, and evaluating outcomes that emphasize a combination of CSF biomarkers (Gisslen et al. 2007). There are three biomarkers related to cardinal components of HIV-related neurodegenerative disorders: CNS HIV infection (measurement of CSF HIV RNA), intrathecal immunoactivation (CSF neopterin), and brain injury (CSF light chain neurofilament).

Careful analysis of this and other biomarker combinations promises more rational trial design and more rapid progress in managing CNS HIV infections as well as its neurological sequelae with antiviral approaches.

CSF sAPPα and sAPPβ concentrations are highly correlated and reduced in patients with AIDS dementia complex (ADC) and opportunistic infections compared to the other groups (Gisslen et al. 2009). Parallel reductions of CSF sAPPα and sAPPβ in ADC and CNS opportunistic infections suggest an effect of CNS immune activation or inflammation on neuronal amyloid synthesis or processing. Elevation of CSF t-tau in some ADC and CNS infection patients without concomitant increase in p-tau indicates neural injury without preferential accumulation of hyperphosphorylated tau as found in Alzheimer's disease. These biomarker changes define pathogenetic pathways to brain injury in ADC that differ from those of Alzheimer's disease.

CSF Kynurenic Acid Level as a Biomarker of Tick-Borne Encephalitis

Kynurenic acid (KYNA) is a neuroactive metabolite of tryptophan that is involved in regulation of cognitive functions. Levels of KYNA increase during virus infection and this metabolite interacts with the immune system. A study has analysed CSF KYNA by using high-performance liquid chromatography in patients with tick-borne encephalitis (TBE), a viral infectious disease associated with long-term cognitive impairment (Holtze et al. 2012). Concentrations of CSF KYNA were found to be considerably higher in TBE patients (5.3 nM) than in control subjects (0.99 nM). KYNA concentration in the CSF varied greatly among individuals with TBE, and increased with the severity of disease. Raised levels of the KYNA are a biomarker of TBE and might play a part in the pathophysiology of the disease. A detailed knowledge of endogenous brain KYNA during the course of CNS infection might yield further insights into the neuroimmunological role of the compound and may also provide new pharmacological approaches for the treatment of cognitive symptoms of the disease.

Biomarkers of Epilepsy

With so many different types of seizures and causes of epilepsy, there are no universal biomarkers except EEG measurements. Some biomarkers detect diseases that manifest in seizures. There are no characteristic biomarkers of idiopathic epilepsy except those for monitoring seizures and response to treatment. Epilepsy prevention trials are more complex, lengthy, and costly than standard epilepsy treatment trials for many reasons such as selection of subjects, consent for participation, length of follow-up, and selection of an appropriate endpoint. The use of biomarkers is a possible solution to these. Development of reliable epilepsy biomarkers would be a major advance in management of epilepsy. A classification of biomarkers of epilepsy is shown in Table 14.13.

Table 14.13 Biomarkers of epilepsy

Biomarkers in blood
Fas and bcl-2
High-mobility group box 1
Protein high-mobility group box 1 (HMGB1)
Serum prolactin
Biomarkers in cerebrospinal fluid
Cytkines following seizures
Lactate elevation following seizures
Metabolites in inborn errors of metabolism with infantile epilepsy
Neuron-specific enolase (biomarker for neuronal injury)
S100 protein
Electrophysiological biomarkers
EEG patterns
Gene mutations in genetic epilepsies
MRI biomarkers

Biochemical Markers of Epilepsy

One study shows that biomarkers of apoptosis, both the proapoptotic Fas and the anti-apoptotic Bcl-2, are proportionately elevated in sera of patients with idiopathic epilepsy, and their levels are related to the seizure severity and frequency.

Elevated serum prolactin assay, when measured in the appropriate clinical setting at 10 to 20 min after a suspected event, is a useful adjunct for the differentiation of generalized tonic-clonic or complex partial seizure from psychogenic nonepileptic seizure among adults and older children. Serum prolactin assay does not distinguish epileptic seizures from syncope and its use has not been established in the evaluation of status epilepticus, repetitive seizures, and neonatal seizures.

Pyridoxine dependency is an uncommon but important cause of intractable seizures presenting in infancy and early childhood. It is usually treated by pyridoxine supplementation. Some children with intractable seizures respond to pyridoxal phosphate rather than pyridoxine, including a rare form of neonatal epileptic encephalopathy shown to be due to mutations in the PNPO gene for pyridox(am)ine 5′-phosphate oxidase. Although the biochemical explanation for this finding is not clear, elevated pipecolic acid levels may serve as a diagnostic biomarker for patients with pyridoxine-dependent seizures. Levels of both pipecolic acid and certain metabolites shown to be elevated in patients with PNPO mutations should be measured, and therapeutic trials of pyridoxal phosphate as well as pyridoxine should be considered early in the course of the management of infants and young children with intractable seizures.

Biomarkers of Temporal Lobe Epilepsy

Temporal lobe epilepsy (TLE) is a common form of focal epilepsy. Results of a clinical study suggest that serum biomarkers are predictive of higher frequencies of seizures in the TLE (Chang et al. 2012). HSP70 levels showed an inverse correlation with hippocampal volume after controlling for the effect of age. HSP70 may be considered to be a stress biomarker in patients with TLE in that it correlates inversely with memory scores and hippocampal volume. In addition, the symmetric extratemporal atrophic patterns may be related to damage of neuronal networks and epileptogenesis in TLE.

Biomarkers of Drug-Resistant Epilepsy

Approximately 30% of epilepsy patients do not respond to AEDs, and neuroinflammation plays a pathogenic role in drug-resistant epilepsy. The high-mobility group box 1 (HMGB1)/TLR4 axis is a key initiator of neuroinflammation following epileptogenic injuries, and its activation contributes to seizure generation in animal models. The pathologic disulfide HMGB1 isoform progressively increases in blood before epilepsy onset and prospectively identified animals that develop the disease (Walker et al. 2017). The authors also observed early expression of disulfide HMGB1 in patients with newly diagnosed epilepsy, and its persistence was associated with subsequent seizures. In contrast with patients with well-controlled epilepsy, patients with chronic, drug-refractory epilepsy persistently expressed the acetylated, disulfide HMGB1 isoforms. Moreover, treatment of animals with antiinflammatory drugs during epileptogenesis prevented both disease progression and blood increase in HMGB1 isoforms. These data suggest that HMGB1 isoforms are mechanistic biomarkers for epileptogenesis and drug-resistant epilepsy in humans. They need evaluation in larger-scale prospective studies.

Genetic Epilepsies

Considerable progress has been made in the discovery of genes that influence risk for epilepsy. Genotyping may identify individuals with genetic epilepsies. However, these gene discoveries have been in epilepsies with Mendelian modes of inheritance, which comprise only a tiny fraction of all epilepsy. Most people with epilepsy have no affected relatives, suggesting that the great majority of all epilepsies are genetically complex: multiple genes contribute to their etiology, none of which has a major effect on disease risk.

Electrophysiological Biomarkers of Epilepsy

Pathological changes in excitability of cerebral cortex usually underlie the initiation and spread of seizure activity in epilepsy. Therefore, monitoring of excitability and controlling its degree using antiepileptic drugs (AEDs) is important for management of epilepsy. Insights into ongoing cortical activity have identified global levels of phase synchronization as measures that characterize normal levels of excitability as well as quantify any deviation therefrom. The usefulness of these intrinsic measures to quantify cortical excitability in humans has been explored (Meisel et al. 2015). The authors observed a correlation of such biomarkers with stimulation-evoked responses and tested them on long-term electrocorticogram and EEG recordings, which indicates that they are viable excitability measures. They reported a significant covariation with the level of AED load and a wake-dependent modulation. The results indicate that excitability in epileptic networks is effectively reduced by AEDs and suggest the proposed biomarkers as useful candidates to quantify excitability in routine clinical conditions overcoming the limitations of electrical or magnetic stimulation. The wake-dependent time course of these biomarkers suggests a homeostatic role of sleep, to rebalance cortical excitability.

Imaging Biomarkers of Epilepsy

Quantitative measurements by MRI of overall brain volume (gray matter, white matter, and CSF) in temporal lobe epilepsy are clinically meaningful biomarkers that are associated with increased cognitive morbidity. Focal cortical dysplasia (FCD) is a common cause of pharmacoresistant epilepsy that is amenable to treatment by surgical resection. The identification of structural FCD by MRI can contribute to the detection of the epileptogenic zone and improve the outcome of epilepsy surgery. New magnetic resonance-based techniques, such as MR spectroscopy, fMRI, and fMRI/EEG, are more frequently being used to increase the yield of MRI in detecting abnormalities associated with epilepsy.

High-magnetic-field MRI and long-term video EEG in a rat model of febrile status epilepticus (FSE) has revealed that reduced amygdala T2 relaxation times can predict TLE (Choy et al. 2014). Reduced T2 values likely represent paramagnetic susceptibility effects derived from increased unsaturated venous hemoglobin, suggesting augmented oxygen utilization after FSE termination. Use of deoxyhemoglobin-sensitive MRI sequences enabled visualization of the predictive changes on lower-field, clinically relevant scanners. This novel MRI signature represents a predictive biomarker for early identification of FSE individuals who are likely to develop TLE and are candidates for preventive therapy.

Noninvasive imaging of brain inflammation would be helpful in determing its role in epileptogenesis and serve as a biomarker for epilepsy. The current imaging

toolbox is limited by the range of neuroinflammatory targets that can be visualized directly. Research in this area will further advance as highly specific ligands and reproducible as well as practical imaging approaches become available (Amhaoul et al. 2014).

Protein Biomarkers of Inflammation in Epilepsy

Molecular and functional interactions between high mobility group box-1 (HMGB1) and the N-methyl-d-aspartate receptor (NMDAR), two proteins playing a key role in neuronal hyperexcitability, have been studied in primary cultures of mouse hippocampal neurons (Balosso et al. 2014). HMGB1 normally resides in the nucleus to regulate transcription, but translocates to the cytoplasm in response to cellular injury and is released into the extracellular milleu where it functions as a pro-inflammatory cytokine biomarker. Disulfide HMGB1 increased phosphorylation of the NR2B subunit of the NMDAR, which is known to increase Ca^{2+} channel permeability and increase NMDA-induced neuronal cell death in vitro and enhance kainate-induced seizures in vivo. This novel molecular neuronal pathway activated by HMGB1 could be targeted in vivo to prevent neurodegeneration and seizures mediated by excessive NMDARs stimulation.

Biomarkers of Normal Pressure Hydrocephalus

Normal pressure hydrocephalus (NPH) or chronic adult hydrocephalus was considered to be a rare condition but recent epidemiological studies indicate that 5–10% of patients suffering from dementia have this condition. Although, surgical diversion of the cerebrospinal fluid (CSF) is the only known procedure to treat the symptoms of this condition, the selection of patients for this procedure has always been problematic. Several diagnostic tests are used for the selection of these patients but the role of biomarkers has so far been overlooked in this condition, as compared to that for other neurodegenerative disorders. Neurochemical biomarkers that could help to separate NPH from other neurological disorders, which mimic NPH symptoms, might assist in the more appropriate selection of patients for CSF shunt surgery.

A review of the research carried out in the last 25 years regarding the identification of serum and CSF biomarkers for NPH concludes that TNF-α, tau protein, lactate, sulfatide and neurofilament triple protein are the most promising CSF markers for NPH. Proteomic studies have shown that CSF level of leucine-rich alpha-2-glycoprotein is a specific biomarker for INPH and has potential use in the diagnosis and indication for CSF shunting. Currently, none of these meet the criteria required to justify a change in clinical practice. In the future, collaborative multicenter projects will be needed to obtain more substantial data that overcome the problems that arise from small uncontrolled studies.

Biomarkers of Pseudotumor Cerebri

Pseudotumor cerebri, also refered to as idiopathic intracranial hypertension (IIH) is an uncommon disorder characterized by increased intracranial pressure without radiological or laboratory evidence of intracranial pathology except empty sella turcica, optic nerve sheath with filled out CSF spaces, and smooth-walled nonflow-related venous sinus stenosis or collapse. IIH typically affects obese women. The incidence of IIH is increasing with the rising prevalence of obesity. Persistent headache is the most common symptom. Visual impairment is a serious complication and papilledema is always present. Pathogenesis of IIH remains unknown. One study has demonstrated the presence of oligoclonal bands in IIH patients that is associated with vision loss (Altıokka-Uzun et al. 2015). Patients with IIH had significantly higher serum levels of TNF-α, IFN-γ, IL-4, IL-10, IL-12, and IL-17 in comparison to multiple sclerosis patients and normal controls. IL-2, IL-4, IL-10, IL-17, and IFN-γ levels in CSF were higher in IIH patients compared to patients with multiple sclerosis or nonorganic/noninflammatory neurologic conditions.

There is a study aimed at identifying the CSF proteome in IIH patients as the new biomarkers (Brettschneider et al. 2011). Six proteins were upregulated in IIH, namely, sterol regulatory element-binding protein 1, zinc-α-2-glycoprotein, immunoglobulin heavy constant α-1, α-1-antitrypsin, serotransferrin, and haptoglobin. Four proteins were downregulated in IIH, including hemopexin, angiotensinogen, vitamin-D-binding protein, and transthyretin. Angiotensinogen was the first protein validated in the study, and it was found that down-regulation of angiotensinogen may contribute to the increased CSF production, which subsequently causes IIH. The study of other proteins may provide more knowledge on the new biomarkers for diagnosis of IIH. Moreover, these proteins may be the target for therapeutic intervention.

Low CSF protein level may have diagnostic utility as a biomarker for prepubertal IIH. Furthermore, this finding suggests that some cases of prepubertal IIH may be caused by CSF overproduction rather than decreased CSF resorption (Margeta et al. 2015).

Biomarkers of Retinal Disorders

Optic nerve and retina are extensions of the nervous system and are included in this chapter. An important disorder is age-related macular degeneration.

Biomarkers of age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a degenerative retinal disease that causes progressive loss of central vision. AMD is the leading cause of irreversible vision loss and legal blindness in individuals over the age of 50. Carboxyethylpyrrole

(CEP) is a biomarker that has been found recently to be associated with susceptibility to AMD. CEP protein adducts are free radical-induced oxidative protein modifi cations generated from docosahexaenoate (DHA)-containing lipids. DHA is easily oxidizable and abundant in ocular tissues where it is exposed to high photooxidative stress. Immunocytochemistry localized CEP to photoreceptor rod outer segments and retinal pigment epithelium in mouse retina. The mean level of CEP adducts are 1.5-fold higher and that of antibody titers in plasma are 2.3-fold higher in patients with AMD compared with normal age-matched controls. Of individuals exhibiting both antigen and autoantibody levels above the mean for non-AMD controls, 92% suffer from AMD. Based on these results, CEP immunoreactivity and autoantibody titer may have diagnostic utility in predicting AMD susceptibility. In the future, serum proteomic pattern analysis will be used to identify additional AMD biomarkers. Being a complex disease, it is possible that multiple biomarkers will be needed to identify those at risk for AMD. The use of signature ion clusters may provide better accuracy and reliability in the process.

The detection of alphaB-crystallin in the retinal pigment epithelium (RPE) of patients with early and advanced AMD implicates this is a biomarker of the disease (De et al. 2007). Changes in the gene expression of RPE cells accompany early stages of the disease and introduces novel potential targets for AMD therapy. In a study on patients with AMD, conventional morphometric measurements correlated with best corrected visual acuity at baseline, but had limited predictive value regarding recovery of visual function. However, optical density ratios (ODR) correlated with best corrected visual acuity under therapy with intravitreous ranibizumab and was the only parameter that was pathognomic for AMD as it reflects the status of the blood-retina barrier and may be used as a biomarker for pathophysiologic differentiation and prognostic purposes in exudative AMD (Ahlers et al. 2009).

High levels of several biomarkers in white blood cells or their protein products in the blood may correlate with the disease state or a predisposition to develop AMD. Using microarray analysis and quantitative real time RT-PCR, a group of genes which show increased expression in white blood cells of patients with AMD have now been identified to facilitate its diagnosis at an early stage when ophthalmoscopic findings are normal.

CRP is produced by the liver and, similar to CEP, can be detected in blood serum by using an immunological test. It is present during episodes of acute systemic inflammation, and higher levels of CRP have been associated with higher risk of developing AMD. A twofold increased risk of AMD is associated with the highest levels of CRP for both smokers and non-smokers. Elevated CRP suggests that inflammation may play a role in the pathophysiology of AMD and therefore antiinflammatory agents might be useful for delaying the disease.

Although several studies have demonstrated strong genetic associations between AMD and SNPs in a number of genes, other modes of regulation are also likely to play a role in the etiology of this disease. A significantly decreased level of methylation was identified on the IL17RC promoter in AMD patients (Wei et al. 2012). Furthermore, hypomethylation of the IL17RC promoter in AMD patients was shown to lead to an elevated expression of its protein and mRNA in peripheral blood

as well as in the affected retina and choroid, suggesting that the DNA methylation pattern and expression of IL17RC may potentially serve as a biomarker for the diagnosis of AMD and likely plays a role in disease pathogenesis.

Biomarkers of Sleep Disorders

Sleep disorders are common, e.g., insomnia. Some of the sleep disorders are secondary to diseases of other systems and primary sleep disorders can also produce neurological dysfunction as well as other systemic changes.

Biomarker of Excessive Daytime Sleepiness

Sleep restriction can produce cognitive deficits and is associated with increased risk for traffic and occupational accidents. Currently, there is no simple quantifiable biomarker that can detect an individual who is excessively sleepy before adverse outcomes become evident. Studies using genetic and pharmacological tools that dissociate sleep drive from wake time in the model organism *Drosophila melanogaster* have identified a biomarker, Amylase, that is highly correlated with sleep drive. Both salivary Amylase activity and mRNA levels are also responsive to extended waking in humans. These findings indicate that the fly is relevant for human sleep research and represents a first step in developing an effective biomarker for detecting sleepiness in vulnerable populations.

Biomarkers of Obstructive Sleep Apnea

Obstructive sleep apnea (OSA) is associated with cardiovascular morbidity and mortality and many other physiological and immunological disorders. An increase in hypoxia due to OSA may cause generation of reactive oxygen species (ROS). ROS are toxic to biomembranes and may lead to peroxidation of lipids. Studies of biomarkers associated with oxidative stress and inflammation do not support the hypothesis that OSA is linked to increased oxidative stress and decreased antioxidant defense, but they suggest that systemic inflammation characterizes OSA patients. Some of the biomarkers of OSA are associated with comorbidities.

OSA is a major public health problem affecting 2% to 3% of children. The standard diagnostic procedure for establishing the presence of OSA is the overnight polysomnography, which is expensive and not practical for extensive use particularly in children. There is a need for biomarkers of OSA but none have been valdidated yet. An extensive review of literature on this topic concluded as follows (Canto Gde et al. 2015):

- Only the combination of kallikrein-1, uromodulin, urocortin-3 and orosomucoid-1 appears to provide sufficient accuracy to be considered a potential OSA diagnostic biomarker in children.
- In adults, IL-6 and IL-10 appear to exhibit a favorable profile as biomarkers aiming to discriminate patients with and without OSA.

This systemic review was performed to identify biomarkers, which were only detected by chromatography and/or mass spectrometry (MS) and to discuss the role of these biomarkers in the field of OSA (Xu et al. 2015). Numerous proteins and metabolites, including lipid profiles, adrenergic/dopaminergic biomarkers and derivatives, amino acids, oxidative stress biomarkers, and other micromolecules were identified in patients with OSA. Applying chromatography and/or MS methods to detect biomarkers helps develop an understanding of OSA mechanisms. More proteomic and metabolomic studies are warranted to develop potential diagnostic and clinical monitoring methods for OSA.

Biomarkers of Restless Legs Syndrome

Restless legs syndrome (RLS) is a sensorimotor disturbance with features of both neurologic and sleep disorders. Those afflicted describe an intensely uncomfortable, overwhelming urge to move the legs, predominantly in the evening or at night, that is present at rest and relieved only temporarily by movement. Clinically significant RLS is common with a prevalence of 2.7%. It is underdiagnosed, and significantly affects sleep and quality of life. Periodic leg movements in sleep have a prevalence of 4–11% in adults. In the elderly, periodic leg movements are also common in subjects without sleep disturbances. In sleep studies, periodic leg movements are found most frequently in RLS and often occur in narcolepsy, sleep apnea syndrome and REM sleep behavior disorder.

Some of the earliest descriptions of RLS recognized its strongly familial nature. Linkage studies have shown a number of susceptibility loci for familial RLS. A report shows an association between a sequence variant in chromosome 6p and periodic limb movements in sleep in distinct Icelandic and American cohorts of subjects with RLS and their families (Stefansson et al. 2007). The movements may serve as a heritable biomarker, or endophenotype, for RLS. It might assist with linkage to other relevant susceptibility genes so that polygenic interactions can be discerned. From a diagnostic perspective, the use of periodic limb movements in sleep may improve diagnostic accuracy and provide a more homogeneous RLS phenotype for epidemiology, RLS may have a number of subtypes, some with and some without periodic limb movements in sleep. For example, many, but not all, patients with RLS have an excellent response to dopaminergic therapy, which also nearly eliminates periodic limb movements in sleep. The subtyping of patients with RLS, based on physiology and genotyping, may predict treatment responses to these agents. Another important feature of the study was the association between the SNP

and reduced serum ferritin indexes. Multiple studies that used MRI scans, analysis of CSF, transcranial ultrasonography, and analysis of autopsy specimens showed that patients with RLS had a reduction in iron in the CNS. Iron levels in the brain may thus reflect an independent endophenotype for RLS or may be a causative factor for RLS or periodic limb movements in sleep.

Serum metabolite profiling in two dopamine-related movement disorders, PD and RLS, compared to a large general population sample identified significant alterations in the polyunsaturated fatty acid metabolism in PD and implicated the inositol metabolism in RLS (Schulte et al. 2016). This study was unable to identify a single metabolite or metabolite profile that could fully differentiate individuals with either disease from the general population as well as from each other. Nevertheless, it provides new perspectives on factors potentially involved in bringing about the two diseases as well as possible points of therapeutic intervention.

Biomarkers of Pain

Pain itself is a biomarker of many diseases but there is lack of validated biomarkers of chronic pain syndromes. There is need for such biomarkers to guide analgesic development. Investigations into potential biomarkers for chest pain showed that cardiac biomarkers used to aid in diagnosis and prognosis of cardiac disease correlate with tissue damage rather than with pain (Marchi et al. 2009). Further studies are needed to gain insights into biomarkers for pain to enhance pain management practices.

Currently functional magnetic resonance imaging (fMRI) is is only reliable biomarker of pain. Activation of brain areas involved in pain can be visualized in response to painful stimuli and action of analgesics can be assessed. fMRI has been used to objectively evaluate acupuncture for pain.

Biomarkers of Disorders with Musculoskeletal Pain

Musculoskeletal pain conditions, such as fibromyalgia and low back pain, tend to coexist in affected individuals and are characterized by a report of pain greater than expected based on the results of a standard physical evaluation. There is lack biological markers for accurate diagnosis of these conditions. Genetic polymorphisms reproducibly linked with musculoskeletal pain are found in genes contributing to serotonergic and adrenergic pathways. Elucidation of the biological mechanisms by which these biomarkers contribute to the perception of pain in these patients will enable the development of better diagnostic methods and more effective drugs to facilitate personalized management of pain (Diatchenko et al. 2013).

Biomarkers of Neuropathic Pain

Although there are changes in the nervous system in neuropathic pain, it is difficult to identify biomarkers in blood and peripheral tissues. There is evidence of CNS involvement in neuropathic pain and movement disorders in patients with complex regional pain syndrome (CRPS). Elevated cerebrospinal fluid (CSF) levels of IL-1β and IL-6 have been reported in CRPS patients with and without movement disorders but other studies have failed to replicate these findings. Cystatin C levels in CSF is a predictive biomarker for postherpetic neuralgia in patients with varicella-zoster virus infection.

Use of proteomic technologies to study proteins that are involved into the pathogenesis of nerve injury and neuropathic pain might enable a better understanding of the pathophysiologic signaling pathways in this impairment and facilitate the discovery of specific biomarkers. Validation of histologic and other biomarkers will provide the foundation for research advances, and new clinical trial designs will allow better discrimination of beneficial treatments for neuropathic pain.

Brain Insular Glutamate as Biomarker of Fibromyalgia

Fibromyalgia (FM) is a chronic widespread painful condition that is thought to arise from augmentation of central neural activity. Glutamate (Glu) is an excitatory neurotransmitter that functions in pain-processing pathways. A study was carried out to investigate the relationship between changing levels of Glu within the insula and changes in multiple pain domains in patients with FM (Harris et al. 2008). Proton magnetic resonance spectroscopy (H-MRS) and fMRI examinations were conducted before and after a nonpharmacologic intervention to reduce pain. During H-MRS, the anterior and posterior insular regions were examined separately using single-voxel spectroscopy. The levels of Glu and other metabolites were estimated relative to levels of creatine (Cr) (e.g., the Glu/Cr ratio). During fMRI, painful pressures were applied to the thumbnail to elicit neuronal activation. Experimental pressure-evoked pain thresholds and clinical pain ratings were also assessed prior to each imaging session. Both experimental pain and clinical pain were reduced following treatment. Changes from pre- to posttreatment in Glu/Cr were negatively correlated with changes in experimental pain thresholds and positively correlated with changes in clinical pain. It was concluded that changes in Glu levels within the insula are associated with changes in multiple pain domains in patients with FM. Thus, H-MRS data may serve as a useful biomarker and surrogate end point for clinical trials of FM.

Biomarkers of Visceral Pain

There is a need for predictive biomarkers to test novel experimental medicines in functional gastrointestinal disorders. With visceral pain models, the large coefficient of variation in sensation end points in human studies precludes definitive

conclusions such as go/no go decisions or dose selection for phase IIb or III studies, unless very large numbers of patients are evaluated in phase IIA pharmacodynamic studies. This renders such pharmacological studies ambitious, or unachievable in a timely fashion. Moreover, the results of tests and clinical trials should be interpreted with greater knowledge of the drug pharmacokinetics, including the influence of CYP metabolism and potential drug interactions. Thus, it is important to identify valid biomarkers of visceral pain for the assessment of treatment response in pharmacodynamic studies.

At present, there is no clear evidence that there are effective biomarkers for visceral pain. The pharmacological agents that have been available for testing to date have been able to demonstrate only modest changes in these sensory end points. The exceptions are single studies of high dose fentany and octreotide, which showed magnitudes of change that would be demonstrable with a reasonable number of participants, that is, at least 20. However, a study of fentanyl recorded the fact that ~70% of participants identified that they were on fentanyl and that participants reported alterations in performance. Moreover, it is conceivable that the local irritant effect of octreotide at the site of injection may have unblinded the study or caused a competing somatic pain that interfered with the appraisal of visceral sensation.

Rectal sensitivity and brain imaging studies seem too laborious and impractical to be useful as pharmacodynamic models. It is still unclear whether the foci activated in the brain are specific to irritable bowel syndrome (IBS). Studies contrasting liminal, subliminal and supraliminal stimuli of the rectum and an acoustic control stimulus show that activation of higher emotional centers may more closely reflect the psychological state than being a true center associated with functional gastrointestinal diseases. It is unclear whether the activated brain centers provide a true reflection or biomarker of IBS or visceral pain.

Biomarkers of Migraine

Migraine patients have chronically low systemic seotonin (5-HT), predisposing them to develop migraine. During the attack, 5-HT levels rise due to increased release from the platelets but selective stimulation of 5-HT1 receptors can abort the attacks. An important role has emerged for neuropeptides, neuronal receptors and neurogenic inflammation in the pathophysiology of migraine. Dilatation of blood vessels in the dural covering of the brain is mediated via release of calcium gene-related peptide (CGRP). This forms the basis of exploration of use of CRGP for counteracting the inflammatory effect and reducing the vascular dilatation for relief of headache. Matrix metalloproteases (MMPs) have been reported to be elevated in blood during migraine attacks but this is a secondary phenomenon and nonspecific as several other disorders, neurological as well as non-neurological, have elevations of MMPs.

Results of the Dutch CAMERA 1 (Cerebral Abnormalities in Migraine, an Epidemiologic Risk Analysis) study show that levels of the CRP protein, as measured on high-sensitivity CRP (hsCRP) assay, are higher by 11% in persons with migraine compared with those in unaffected individuals, rising to 17% among women (Tietjen et al. 2017). Fibrinogen and Factor II were associated with migraine aura in women but not men. In the migraine subgroup, the total number of years of aura, but not headache, predicted elevated hs-CRP, and the average number of aura, but not headache attacks, predicted all biomarkers with exception of Factor II.

Biomarkers of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

Chronic fatigue syndrome (CFS) has been defined as clinically evaluated, unexplained, persistent or relapsing chronic fatigue that is of new or definite onset, is not the result of ongoing exertion, is not substantially alleviated by rest, and results in substantial reduction in previous levels of occupational, educational, social, or personal activities. Sore throat, fever, tender lymph nodes, general weakness, and muscle pain are the most frequent symptoms. In view of more recent research and clinical experience that strongly point to widespread inflammation and multisystemic neuropathology, International Consensus Panel has labeled CFS as "myalgic encephalomyelitis"(ME) because it indicates an underlying pathophysiology (Carruthers et al. 2011). More than 2 million persons in the US have ME/CFS.

There is no validated biomarker for ME, but hyperactivation of melanotrophs in the pituitary gland and increased levels of plasma alpha-melanocyte-stimulating hormone (α-MSH) have recently been reported in an animal model of chronic stress. Because ME is considered to be caused partly by chronic stress, increased α-MSH plasma levels may also occur in these patients. A study on ME patients with a disease duration of up to 5 years had significantly higher levels of α-MSH in their peripheral blood (Shishioh-Ikejima et al. 2010). Although raised α-MSH levels are also observed in congestive heart failure, obesity, and inflammatory diseases such as sepsis and HIV, all these diseases can be diagnosed and excluded in patients with ME. α-MSH could be a potent biomarker for the diagnosis of ME, at least during the first 5 years after onset of the disease.

There is now evidence that specific subgroups of patients with ME/CFS suffer from a neuropsychiatric immune disorder. Results of a study that measured peripheral lymphocytes expressing CD3+, CD19+, CD4+, CD8+, CD38+ and HLA-DR+ in ME/CFS and normal controls support the following statements (Maes et al. 2015):

- Increased CD38 and HLA-DR expression on CD8+ T cells are biomarkers of ME/CFS.
- Increased CD38 antigen expression may contribute to suppression of the CD4+/CD8+ ratio and CD19+ expression.

- There are different immune subgroups of ME/CFS patients, e.g. Increased CD8+ activation biomarker expression versus inflammation
- Viral infections or reactivation may play a role in a some ME/CFS patients.

A targeted metabolic study of 63 biochemical pathways by hydrophilic interaction LC, ESI, and tandem MS on plasma from patients with CFS showed abnormalities in 20 metabolic pathways with 80% decrease in diagnostic metabolites, consistent with a hypometabolic syndrome (Naviaux et al. 2016). Pathway abnormalities included sphingolipid, phospholipid, purine, cholesterol, microbiome, pyrroline-5-carboxylate, riboflavin, branch chain amino acid, peroxisomal, and mitochondrial metabolism. Diagnostic accuracies were 94% in males using 8 metabolites and 96% in females using 13 metabolites. These data show that despite the heterogeneity of factors leading to CFS, the cellular metabolic response in patients was homogeneous, statistically robust, and chemically similar to the evolutionarily conserved persistence response to environmental stress known as dauer (German word for persistence or long-lived). The study shows that CFS has an objectively identifiable "chemical signature" or biomarker in both men and women and targeted metabolomics, which provide direct small molecule information, can provide actionable treatment information. Only 25% of the metabolite disturbances found in each person were needed for the diagnosis of CFS. Approximately 75% of abnormalities were unique to each individual, which is useful in guiding personalized treatment. A criticism of this study is that metabolic differences between patients with CFS and healthy controls do not automatically yield a test that is diagnostic of CFS (Vogt et al. 2016).

Biomarkers of Psychiatric Disorders

Psychiatric disorders are still being diagnosed on the basis of symptoms and behavioral observation without corresponding biological validation. This contrasts with other fields of medicine, where diagnosis and treatment are based not only on clinical examination, but also biological tests based on validated biomarkers. There is now an increasing interest in conducting studies for biomarker identification in psychiatric disorders.

Anorexia Nervosa

Anorexia nervosa is an eating disorder characterized by an obsessive fear of gaining weight and an inability to stay at the minimum body weight considered healthy for the person's age and height. Most of the laboratory investigations are done to rule out other diseases and assess the effects of malnutrition on various body systems. Even the most critically ill anorexic patients may present with normal 'standard' laboratory values, underscoring the need for a sensitive biomarker.

A prospective cohort study on patients with severe anorexia nervosa, as defined by a body mass index <14 kg/m^2, showed that serum complement C3 levels were significantly lower in patients with anorexia nervosa than in controls (Flierl et al. 2011). Thus C3 serum levels may represent a sensitive new biomarker for monitoring the severity of disease in anorexia nervosa.

Attention-Deficit Hyperactivity Disorder

Attention-deficit hyperactivity disorder (ADHD) is mostly a childhood psychiatric disorder although it may occur in adults as well. ADHD manifests with symptoms of inattention, hyperactivity and/or impulsivity that manifests in the family, the school and in social interactions. ADHD is frequently misdiagnosed and the increasing use of neuroimaging techniques in ADHD research have led to consideration of an imaging test for the diagnosis of ADHD that could be useful in clinical practice. A consistent decrease in volume of right caudate nucleus in ADHD could provide a good basis for any future diagnostic biomarker (Soliva 2011).

A meta-analysis of studies that used proton magnetic spectroscopy to study ADHD revealed that most studies have focused on the frontal lobe and the basal ganglia (Perlov et al. 2008). Relative to creatine, choline compounds, N-acetylaspartate, and glutamate/glutamine were altered in ADHD. Meta-analytic techniques showed that children showed significant changes of choline compounds in left striatum and right frontal lobe. Other studies in adults have implicated the cerebellum (Perlov et al. 2010; Soliva et al. 2010).

In a prospective study, brain iron was indexed noninvasively by using MRI imaging relaxation rates and magnetic field correlation (MFC) in the globus pallidus, putamen, caudate nucleus, and thalamus of patients with ADHD, both treatment-naïve and those treated with psychostimulants (Adisetiyo et al. 2014). Serum iron values were also recorded. Lower MFC indexes of striatal and thalamic brain iron in medication-naïve ADHD patients and lack of differences in psychostimulant-medicated patients suggest that MFC indexes of brain iron may represent a noninvasive diagnostic biomarker that responds to psychostimulant treatment.

Biomarkers of Autism

Autism is a neuropsychiatric disorder that appears during the first 3 years of life and impairs normal development. Children and adults with autism spectrum disorder (ASD) have difficulty in verbal and nonverbal communication, social interaction and imaginative activities including play. ASD is one of the most common developmental disabilities. Global prevalence of ASD is estimated to be 17 per 10,000 persons (Elsabbagh et al. 2012). According to the CDC, there has been a significant rise

Table 14.14 Biomarkers of autism spectrum disorder

Disturbances of gastrointestinal microbiota
Epigenetic biomarkers: histone acetylation patterns
Gene polymorphisms
Genetic factors
Immune biomarkers
Metabolic disturbances
Neurophysiological biomarkers
Oxidative stress biomarkers
Gene expression panel
Umbilical cord biomarkers

in the prevalence of ASDs over the past decade, from ~0.2% of the US population to 2% of school age children.

Currently, the diagnosis of ASD is based solely on the presence of a complex phenotype as assessed by a qualified professional. Table 14.14 shows several biomarkers associated with ASD; none of these has proven to be useful as a screening test or for clinical diagnosis.

Epigenetics of ASD

Epigenetic profiling has shown that despite tremendous heterogeneity in the primary causes of autism, such as DNA mutations and environmental perturbations during development, ASD has molecular features that are commonly shared. Examination of post-mortem brain samples from individuals with or without ASD suggest that histone acetylation patterns in the brain's cortex are similar across majority of individuals with ASD, which opens the possibility of designing drugs to correct these changes (Sun et al. 2016). This study used ChIP-seq with the Illumina HiSeq 2000, targeting H3K27ac, a histone acetylation biomarker, which has been linked to active gene promoters and enhancers. The histone acetylation signature described so far was not found in every ASD case, but at least 80% of individuals with the condition had similar H3K27ac biomarkers at >5000 regulatory sites in the genome.

Gastrointestinal Microbiota Disturbances and ASD

Many individuals affected with ASDs also display symptoms of gastrointestinal (GI) disturbance, suggesting GI factors may play an important role in the pathogenesis of ASD and/or related complications. There is evidence supporting a role for

the GI microbiota and their fermentation products in the etiology and/or symptoms of ASD, and their potential use as biomarkers (Wang et al. 2014). Levels of gut *Clostridia, Bacteroides, Bifidobacteria* and *Akkermansia spp.*, have been observed to be abnormal in individuals with ASD. In addition, gut fermentation products including short-chain fatty acids, ammonia and p-cresol may play a role. GI-related biomarkers could potentially enable early identification of ASD at risk of GI disturbance, and thereby guide targeted interventions, potentially improving the health and quality of life of affected individuals.

Genetic Factors in ASD

Genetics of ASD is be rather complex as only ~10% of the cases can be accounted for by mutations in numerous genes or in association with known syndromes such as fragile X and tuberous sclerosis. An equal percentage of patients carry CNVs (microdeletions or duplications), some of which also exist at a lower frequency in the control population. In addition, the vast majority of individuals with ASD have no strong family history for the disorder indicating that ASD is a complex condition for which simple Mendelian genetics will not provide a simple answer.

Immune Biomarkers of ASD

Maternal infections and inflammation have known links with ASD. Maternal autoantibodies have the ability to cross the placenta into the fetal circulation as well as the BBB and combine with antigens to create immune complexes capable of damaging neurological tissue of the fetus (Elamin and Al-Ayadhi 2014). Autoantibodies can be used as novel biomarkers for autism and also provide insights into the neurodevelopmental processes that lead to autism.

Metabolic Disturbances in Autism

ASDs are associated metabolic derangements. A study has aimed to identify a pattern of metabolic perturbance in ASD using metabolomics in urinary specimens from children with ASD and age matched controls (Ming et al. 2012). Using a combination of liquid- and gas-chromatography-based MS, the levels of 82 metabolites (53 of which were increased) were detected that were significantly altered between the ASD and the control groups using osmolality normalized data. Pattern analysis showed that the levels of several amino acids such as glycine, serine, threonine, alanine, histidine, glutamyl amino acids and the organic acid, taurine were significantly lower in ASD children. The levels of antioxidants such as carnosine were also reduced in ASD. Furthermore, several gut bacterial metabolites were significantly altered in ASD children who had gastrointestinal dysfunction. Overall, this study detected abnormal amino acid metabolism, increased oxidative stress, and altered

gut microbiomes in ASD. The relationship of altered gut microbial co-metabolism and the disrupted metabolisms requires further investigation.

Elevated levels of uric acid have been reported in the urine of some autistic persons. Uric acid is the end product of purine metabolism and is elevated in other diseases of purine metabolism such as Lesch-Nyhan Syndrome. Patients with hyperuricosuric autism benefit from metabolic therapy with oral uridine therapy in a manner similar to that seen in other disorders of purine metabolism in which there is autistic symptomatology. Some studies did not reveal significant difference in the levels of xanthine and methylxanthines in the urine samples from patients diagnosed with autistic symptoms suggesting that they are uncommon manifestation of autism. There is no reliable biomarker of autism based on uric acid metabolism.

Use of Phenotype MicroArray platform (Biolog) to profile multiple metabolic pathways in individuals with various neurodevelopmental disorders has revealed a significant decrease in the utilization of tryptophan as an energy source in cell lines from individuals with ASD, which also supports a proposed possible mitochondrial dysfunction in cells of patients with ASD that can affect synaptic plasticity and neuronal development. Although only a small fraction of tryptophan is metabolized along the serotonin-melatonin pathway, it is important for the generation of serotonin – an important neurotransmitter because of its involvement in multiple brain functions. Finally, the measurement of serotonin levels has been the most consistent biomarker for ASD. It is quite possible that impairment of the metabolism of tryptophan, by any one of numerous means, provides the unifying model that explains the heterogeneity of ASD and the difficulty in identifying a universal biomarker (Schwartz 2014). Measurement of the decrease in tryptophan metabolism in cells from patients may provide a reliable screening test for ASDs.

NeuroPointDX, through its sister division Stemina Biomarker Discovery, has developed the leading metabolomics platform for identifying differences in metabolism of patients with ASD and other neurological disorders. Metabolic biomarkers are being validated in a clinical trial.

Neurophysiological Biomarkers

The excess of high frequency oscillations, γ-band abnormalities, as observed on EEG and magneto-encephalography (MEG), may reflect imbalance in the excitation-inhibition homeostasis in the cortex. Given the important role of high frequency EEG rhythms for perceptual and cognitive processes, early and probably genetically determined abnormalities in the neuronal mechanisms generating high frequency EEG rhythms may contribute to development of ASD (Orekhova et al. 2007). γ-band are potential biomarkers of ASDs as observed in studies using various stimuli in affected individuals as well as unaffected first-degree relatives (Rojas and Wilson 2014). Abnormalities in oscillatory responses to language were seen in parents of subjects with ASD that are consistent with previous findings in ASD probands and these findings are supportive of γ-band activity as a heritable, neurophysiological biomarker of ASD (McFadden et al. 2012). The possible

relationship seen between language function and neural activity in this study should be investigated further to assess if oscillatory response abnormalities may contribute to behavioral manifestations. Associations between γ-band abnormalities and clinical severity can be used as both predictors of diagnosis and as surrogate biomarkers in clinical trials of new interventions for ASD.

Role of Oxidative Stress in Autism

The exact cause of autism is not known but it could result from an interaction between genetic and environmental factors with oxidative stress as a potential mechanism linking the two. One genetic factor may be altered oxidative-reductive capacity. High levels of homocysteine and oxidative stress are generally associated with neuropsychiatric disorders. Children with have higher levels of total homocysteine, which is negatively correlated with glutathione peroxidase activity, low paraoxonase 1 arylesterase activity and suboptimal levels of vitamin B12. There is a trend towards an increase in levels of 8-OHdG, a biomarker of stress, in children with autism but it does not reach statistical significance. However, lipid peroxidation biomarker is increased in autistic children.

Test for ASD Based on a 55-Gene Expression Panel

Based on the results of the largest blood transcriptome study to date that aims to identify differences in ASD cases and 115 age/sex-matched controls, prediction model was developed, which achieved 68% classification accuracy with the validation cohort (Kong et al. 2012). The blood-based test appears to predict autism relatively accurately, at least among boys. The results suggest that the use of blood expression profiling for ASD detection may be feasible. Further study is required to determine the age at which such a test should be deployed, and what genetic characteristics of ASD can be identified. The test has already been licensed to SynapDx for commercial development.

Umbilical Cord Biomarkers

Potential umbilical cord biomarkers including IGF, anti-myelin basic protein (MBP) and serotonin (Steinman and Mankuta 2014). IGF is essential for the myelination of developing fetal neurons. IGF with well-known links to maternal inflammation, infection and ASD, is a potential biomarker and is currently being investigated in prospective studies. Combination of IGF data with that of the known biomarkers – serotonin and anti-MBP – in order to calculate an autism index, could provide a new diagnostic method for at-risk neonates

Biomarkers of Bipolar Disorder

Bipolar disorder (BD) is a mood disorder characterized by mania alternating with depression. It may vary in severity from mild form, hypomania, to severe psychosis. Diffusion tensor imaging studies (DTI) of BD show abnormalities in fractional anisotropy (FA) and radial diffusivity (RD), measures of the integrity of white matter (WM), which may reflect underlying pathophysiologic processes. There is, however, a need to identify peripheral measures that are related to these WM measures, to help identify easily obtainable peripheral biomarkers of BD. Given the high lipid content of axonal membranes and myelin sheaths, and that elevated serum levels of lipid peroxidation are reported in BD, these serum measures may be promising peripheral biomarkers of underlying WM abnormalities in BD. A study has used DTI and probabilistic tractography to compare FA and RD in prefrontal-centered WM tracts, most of which consistently show abnormal FA (and/or RD) in BD, and also examined serum lipid peroxidation in currently euthymic BD adults and gender-matched healthy adults (Versace et al. 2014). There was a significant effect of group upon FA in these a priori WM tracts and RD and a significant between-group difference in lipid hydroperoxides (LPH). Multivariate multiple regression analyses revealed that LPH variance explained, respectively, 59 and 51% of the variance of FA and RD across all study participants. This is the first study to examine relationships between measures of WM integrity and peripheral measures of lipid peroxidation. These findings suggest that serum LPH may be useful in the development of clinically relevant, yet easily obtainable and inexpensive, peripheral biomarkers of BD.

Multiplex profiling of 320 proteins utilizing the Myriad RBM Discovery Multi-Analyte Profiling platform was performed on blood samples of a consecutive series of patients with a confirmed diagnosis of unipolar or bipolar depression as well as controls (Frye et al. 2015). After correcting for multiple testing and adjusting for covariates, growth differentiation factor 15 (GDF-15), hemopexin (HPX), hepsin (HPN), matrix metalloproteinase-7 (MMP-7), retinol-binding protein 4 (RBP-4) and transthyretin (TTR) all showed statistically significant differences among groups. MMP-7 was significantly different in mood disorder vs controls, MMP-7, GDF-15, HPN were significantly different in bipolar cases vs controls, and GDF-15, HPX, HPN, RBP-4 and TTR proteins were all significantly different in bipolar cases vs controls. Good diagnostic accuracy was obtained most notably for GDF-15, RBP-4 and TTR when comparing bipolar vs controls. Although based on a small sample that was not adjusted for medication state, the results suggest feasibility of using proteomic panels to assist in identifying and distinguishing mood disorders, in particular bipolar disorder. This study supports the possibility of developing a diagnostic test using the discovered biomarkers, which need to be validated, to help facilitate accurate diagnosis and rapid initiation of treatment with improved clinical outcomes. Further functional studies of the identified proteins will increase our understanding of the pathophysiology of mood disorders, which may lead to the discovery of novel pharmacological targets.

Biomarkers of Depression

Major depressive disorders (MDDs) affect 6.7% of adults in the US each year, and are difficult to diagnose only on the basis of patients' self-reported symptoms. MDD is a heterogeneous disorder with subgroups based on involvement of different systems/ biomarkers such as the dopamine system, the hypothalamic-pituitary-adrenal axis, neuroinflammation, and reduced tryptophan due to the increased activation of the tryptophan-kynurenine pathway (Kunugi et al. 2015). The microbiomes of mice subjected to stress have significantly depleted levels of Lactobacillus leading to reactive oxygen species, which suppress host kynurenine metabolism by inhibiting the expression of the metabolizing enzyme, indoleamine 2,3-dioxygenase, in the intestine (Marin et al. 2017).

Interrelations between various systems should be examined to subtype and integrate the pathophysiology of MDD. Current treatment of MDD is not optimal. Objective biomarkers of subtypes of MDD could increase diagnostic specificity and promote individualized therapy. A scheme of integration of various factors in the pathogenesis of MDD with relevant biomarkers for diagnosis and therapy is shown in Fig. 14.2.

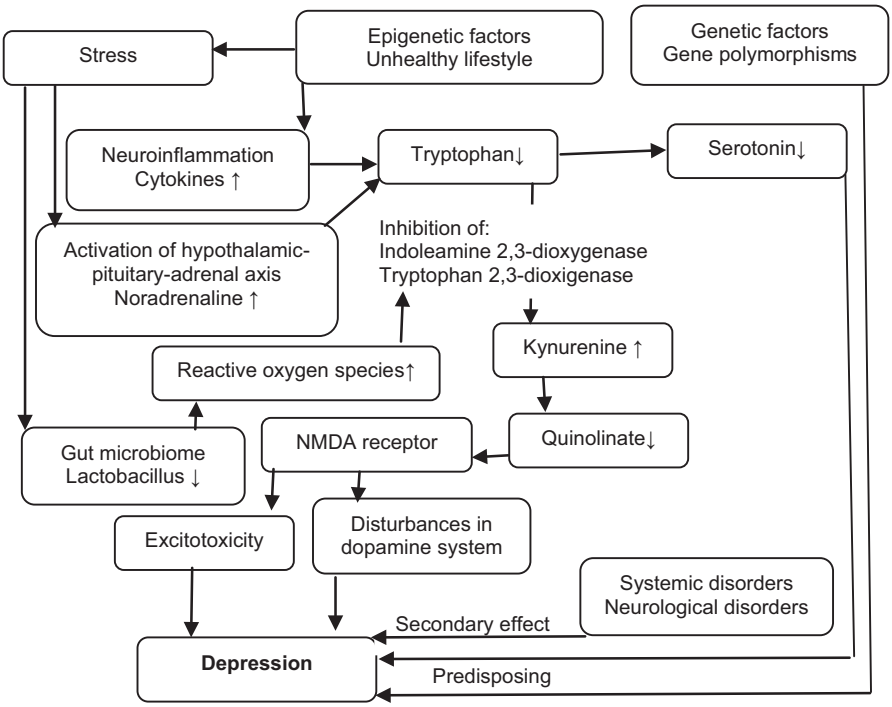

Fig. 14.2 A scheme of pathogenesis of MDD with relevant biomarkers (© Jain PharmaBiotech)

Biochemical Biomarkers of Depression

Altered cytokine secretion as a mechanism in the etiology of depression is still obscure. The serotonin transporter (5-HTT) may play an important role in the termination of serotonergic neurotransmission by serotonin (5-HT) uptaking into presynaptic neurons and representing as an initial action site for selective 5-HTT reuptake inhibitors (SSRI). Cytokines and 5-HTT are potential biomarkers for depression. Various studies show that the mRNA expressions of IL-1β, IL-6, IFNγ, TNFα, and 5-HTT are higher in the depressed patients than those of the healthy controls. The higher level of mRNA expressions of IFNγ and 5-HTT diminish after fluoxetine treatment. Furthermore, a positive correlation is found between 5-HTT and cytokines mRNA expression, which suggests that proinflammatory cytokines and 5-HTT might play critical roles in the pathogenesis of major depression and that their levels were affected by chronic treatment with 5-HTT inhibitors. The most repeatedly validated biomarkers of depression are the decrease in serotonin transporter binding in platelets and lymphocytes, hypercortisolemia, hypocholesteremia, decrease in BDNF, decrease in CREB phosphorylation, and an increase IL-6 (Caruncho and Rivera-Baltanás 2010).

Biomarkers and Response to Antidepressant Treatment

Incorporation of biomarkers in the treatment of MDD could help improve the efficiency of treatment trials and ultimately speed remission (Breitenstein et al. 2014). Biomarkers of response to antidepressant treatment are shown in Table 14.15.

Cingulate Cortex Activity and Response to Antidepressants

Increased rostral anterior cingulate cortex (rACC) activity has emerged as a promising predictor of treatment response in depression, but neither the reliability of this relationship nor the mechanisms supporting it have been thoroughly investigated.

Table 14.15 Biomarkers of response to antidepressant treatment

Biotechnology area	Biomarkers
Brain imaging	Rostral anterior cingulate cortex activity, hippocampal volume
Electrophysiology	Quantitative EEG, REM sleep
Gentic biomarkers	5HTT-LPR determines response to SSRIs
Neuroendocrinology	Dexamethasone/corticotropin-releasing hormone test
Pharmacodynamics	SLC6A, 4HTR2A
Pharmacokinetics	CYP, ABCB1
Proteomics/metabolomics	BDNF, IGF-1, VEGF
Neuroimaging + serum + CSF	Proinflammatory cytokine profile

However, metaanalysis shows that the relationship between resting rACC activity and treatment response is robust, and that the rACC plays a key role in treatment outcome because of its 'hub' position in the default network (Pizzagalli 2011). This hypothesis is supported by neuropsychological, electrophysiological, and neuroimaging data on frontocingulate dysfunction in depression.

Genetic Biomarkers of Response to Antidepressants

Genetic factors alongside environmental variables and gene-environment interactions are implicated in the etiology of mood and anxiety disorders. Genes may influence susceptibility to depression and response to drugs. Several gene polymorphisms are associated with variation of response to antidepressant therapy. 5-HT6 receptor polymorphisms may be associated with response to antidepressant treatment in MDD. The Mayo Clinic offers a test for a genetic biomarker, 5HTTLPR, that identifies people who respond differently to antidepressants, including SSRIs. The serotonin transporter genotype assists the physicians in making a better choice of antidepressant medications for their patients, based upon their serotonin transporter genotype used in conjunction with CYP450 genotyping. Depending upon genotypes, some patients should respond well to SSRIs, some may respond to SSRIs but more slowly, and some patients may respond more effectively to non-SSRI antidepressants.

According to a consensus paper of the World Federation of Societies of Biological Psychiatry, polymorphisms in genes for cytochrome P450 isoenzymes, ABCB1 (antidepressant transport), FKBP5 (glucocorticoid signaling) and serotonin neurotransmission (SLC6A4 and HTR2A), which involved in antidepressant drug metabolism, are included in the tests for predicting response to treatment (Fabbri et al. 2017). Results of clinical studies of these tests are encouraging, but their cost/benefit ratio and indications have not been defined. Future tests may be specific for MDD subtypes and include combinations of different types of biomarkers.

Inflammatory Biomarkers of Depression and Psychosis

Systemic inflammatory biomarkers IL-6 and CRP are linked with the risk of developing heart disease and diabetes mellitus, which are common comorbidities for depression and psychosis. A subsample of individuals from the cohort with data on childhood IL-6 and CRP levels and later psychiatric assessments has been studied in the Avon Longitudinal Study of Parents and Children (ALSPAC) – a prospective general population birth cohort study in England (Khandaker et al. 2014). Higher levels of the systemic IL-6 in childhood were associated with an increased risk of developing depression and psychosis in early adulthood. Inflammatory pathways may provide important new intervention and prevention targets for these disorders.

P11 as a Biomarker of Depression

P11 (protein S100A10) is associated with astrogliosis (Weiss et al. 2016). P11 interacts with serotonin receptors and plays an important role in both the development of depression and therapeutic response to antidepressants (Svenningsson et al. 2013). P11 levels are reduced in depressed individuals in several brain regions, including the frontal cortex, nucleus accumbens, and hippocampus.

Panels of Blood-Based Biomarkers for Diagnosis of MDD

An innovative approach to biomarker discovery for early-onset MDD has combined results from genome-wide transcriptomic profiles in the blood of two animal models of depression, representing the genetic and the environmental, stress-related, etiology of MDD followed by unbiased analyses candidate blood transcriptomic biomarkers in adolescent subjects with MDD (Pajer et al. 2012). A panel of 11 blood biomarkers differentiated participants with early-onset MDD from the normal control group. Additionally, a separate but partially overlapping panel of 18 transcripts distinguished subjects with MDD with or without comorbid anxiety. Four transcripts, discovered from the chronic stress animal model, correlated with poor treatment scores in youths. Pilot data suggest that this approach can lead to clinically valid diagnostic panels of blood transcripts for early-onset MDD, which could reduce diagnostic heterogeneity in this population and has the potential to advance personalized treatment strategies.

A further study has assessed a blood-based biomarker panel, which showed promise in adolescents with MDD, in adult primary care patients with MDD and age-, gender- and race-matched nondepressed controls (Redei et al. 2014). Patients with MDD received cognitive behavioral therapy (CBT) and clinical assessment using self-reported depression with the Patient Health Questionnaire–9 (PHQ-9). A serum-based panel of 9 biomarker (including BDNF, cortisol and soluble TNF-α receptor type II) has shown good sensitivity and specificity in differentiating between MDD and healthy controls. Blood transcript levels of 9 biomarkers – ADCY3, DGKA, FAM46A, IGSF4A/CADM1, KIAA1539, MARCKS, PSME1, RAPH1 and TLR7 – as measured by a qPCR-based approach differed significantly between participants with MDD and non-depressed controls at baseline and after 18 weeks of CBT. Before CBT, significant co-expression network of specific transcripts existed in MDD subjects who subsequently remitted in response to CBT, but not in those who remained depressed. Thus, blood levels of different transcript panels may identify the depressed from the nondepressed among primary care patients, during a depressive episode or in remission, or follow and predict response to CBT in depressed individuals. Transcript patterns of ADCY3, DGKA, IGSF4A/CADM1, PSME1, and RAPH1 at baseline were correlated with remission in response to therapy. This distinction could be used to predict who would respond to the therapy.

Plasma Metabonomics for Diagnosis of MDD

A NMR-based plasma metabonomic method for the diagnosis of MDD was tested on plasma samples from first-episode drug-naive depressed patients and healthy controls (Zheng et al. 2012). Data were recorded and analyzed by orthogonal partial least-squares discriminant analysis and score plots of the spectra demonstrated that the depressed patient group was significantly distinguishable from the healthy control group. Moreover, the method accurately diagnosed blinded samples in an independent replication cohort with a sensitivity and specificity of 92.8% and 83.3%, respectively. Taken together, NMR-based plasma metabonomics may offer an accurate empirical laboratory-based method applicable to the diagnosis of MDD.

Post-Partum Depression

Post-partum depression (PPD) develops in 10 to 15% of all new mothers. Among women previously diagnosed with mood disorders, the rate rises to between 30 and 35%. Early diagnosis and treatment is important before the symptoms of PPD become debilitating. PPD risk is due to an altered sensitivity to estrogen-mediated epigenetic changes that act in a cell autonomous manner detectable in the blood. The hypothesis of relation of estrogen-mediated epigenetic reprogramming events in the hippocampus and predisposition to risk of PPD was investigated using a cross-species translational design (Guintivano et al. 2014). Pathway analyses of data demonstrated that DNA methylation patterns related to hippocampal synaptic plasticity may be of etiological importance in PPD. In a study of measurement of gene expression as well as hormones in the first and third pregnancy trimesters and early postpartum, transcripts that are differentially expressed between the PPD and euthymic women during the third trimester enabled prediction of PPD with an accuracy of 88% (Mehta et al. 2014). These results confirm the previously proposed hypothesis of increased sex-steroid sensitivity as a susceptibility factor for PPD and suggest that PPD can be robustly predicted in currently euthymic women as early as the third trimester.

DNA Methylation Biomarker of Post-Partum Depression Risk Test was developed by scientists at Johns Hopkins University (JHU). The blood-based test detects epigenetic changes in pregnant women and can be used during any trimester, potentially offering a simple method to diagnose PPD in the weeks after a woman has given birth. In 2014, Physician's Choice Laboratory Services licensed this test from JHU to assess its analytical capabilities and potential clinical commercialization.

Biomarkers of Posttraumatic Stress Disorder

Posttraumatic stress disorder (PTSD) can develop following stressful traumatic incidents such as combat, sexual abuse, or natural disasters and manifestations include incapacitating anxiety with the core symptoms of nervous hyperarousal,

Table 14.16 Biomarkers of posttraumatic stress disorder

Biomarkers of risk factors for PTSD
History of psychiatric disorders
Intellectual disability
Low mRNA levels of the FK506 binding protein 5
Multiple trauma
Nightmares
Prone to fear
Reduced levels of estradiol and testosterone
Diagnostic biomarkers
Amygdala hyperactivity
Cognitive impairment
Decrease of hippocampal volume
Dysregulation of hypothalamo-pituitary axis (HPA)
Enhanced startle response
Heart rate: increased heart rate and decreased heart rate variability
Hyperreactivity of sympathetic adrenomedullary system
Immune biomarkers: rise of interleukins (IL-1β, IL-2, IL-6), C-reactive protein, TNF-α, nuclear factor-κB
Metabolic hormones: neuropeptide Y decreased, increased insulin response to glucose
Steroid hormones: allopregnanolone decreased (in women), dehydroepiandrosterone raised
Biomarkers of therapeutic response
Better response to SSRI/SNRI: 5HTTLPR genotype, BDNF serum levels
Better response to CBT: low amygdala activity, high anterior cortex activity
99mTc-HMPAO uptake: differences between responders vs non-responders to EMDR treatment

Abbreviations: *5HTTLPR* serotonin-transporter-linked polymorphic region, *BDNF* brain derived neurotrophic factor, *CBT* cognitive behavioral therapy, *EMDR* Eye Movement Desensitization and Reprocessing, *SSRI/SNRI* selective serotonin reuptake inhibitor/serotonin-norepinephrine reuptake inhibitor

distressing recalls of traumatic memories, and avoidance of trauma-related cues. Incidence of PTSD varies considerably in different populations and countries. It is relatively more common in US war veterans. Individual susceptibility is an important factor in development of PTSD. Currently, there are still no approved biomarkers in clinical use for this debilitating anxiety disorder. Biomarkers of PTSD are shown in Table 14.16.

PTSD is a heterogeneous diagnostic construct with no common pathobiological feature for its varied symptoms; therefore, none of the biomarkers reflect the entire syndrome (Schmidt et al. 2013). Interactions between different biological systems influence biological phenotypes within PTSD. Most promising PTSD biomarker will likely be identified by multidimensional models derived from comprehensive descriptions of molecular, neurobiological, behavioral, and clinical phenotypes (Michopoulos et al. 2015). Examples of these interactions are:

- Gonadal steroid hormones and the HPA axis modulate neurotransmitter and neuropeptide systems, influencing amygdala activity and inflammatory responses.
- HPA activity, via cortisol and CRH (corticotropin releasing hormone), alters sensitivity to gonadal hormones.
- Inflammation alters HPA activity and has adverse effects on cardiovascular function.

Biomarkers of Psychosis

Psychosis is characterized by a loss of contact with reality and is typically associated with hallucinations and delusional beliefs. Among psychiatric conditions that present with psychotic symptoms, schizophrenia is most important, but bipolar affective disorder, and some forms of severe depression can present with psychosis, referred to as psychotic depression. The pathological mechanisms resulting in psychotic symptoms are not understood, nor is it understood whether the various psychotic illnesses are the result of similar biochemical disturbances. The identification of biomarkers of psychosis is fundamental for a better understanding of its pathogenesis has the potential to provide more objective testing methods.

Among various techniques, proteomics, particularly MS, has potential to advance the understanding of the biochemical basis of psychosis and enable development of diagnostics and improved therapeutics. Surface-enhanced laser desorption ionization MS has been used to profile proteins and peptides in CSF samples of psychotic patients and specific protein/peptide changes have been reported. However, further studies are required to validate the clinical relevance and disease specificity of the identified biomarkers.

Biomarkers of Schizophrenia

The current diagnosis of schizophrenia is usually based on the symptoms experienced and reported by the patient, in combination with signs observed by a psychiatrist, clinical psychologist, or other clinician. Biomarkers for early diagnosis of schizophrenia are urgently needed because of lack of objective diagnostic tests. The complex, multidetermined nature of schizophrenia and other psychoses makes it unlikely that any single biomarker will be both sensitive and specific enough to unambiguously identify individuals who will later become psychotic.

Initial studies in schizophrenia, bipolar disorder and major depressive disorder have highlighted the potential utility of multiplex biomarker development. These studies were primarily non-hypothesis driven, based on an established immune mediator and cytokine quantification platforms, and were predominantly compared with a healthy control population. While there has been initial validation, replication and development of classification decision rules in a series of studies in schizophrenia,

the majority of studies were not comparative within mood disorders and were not corrected for multiple testing and adjusted for covariates, thus limiting their replication potential and overall generalizability. Proteomics-based detection of biomarkers for bipolar disorder might enable differentiation of mood disorders from psychoses.

The gold standard for diagnosis of psychosis has been clinical observation, classifying patients into schizophrenia, schizoaffective, and bipolar disorders. A study, the Bipolar-Schizophrenia Network on Intermediate Phenotypes (B-SNIP) used a large biomarker panel (neuropsychological, stop signal, saccadic control, and auditory stimulation paradigms) characterizing diverse aspects of brain function to collected on individuals with schizophrenia, schizoaffective disorder, and bipolar disorder with psychosis, their first-degree relatives, and demographically comparable healthy subjects (Clementz et al. 2016). B-SNIP identified three neurobiologically distinct biotypes that do not always match up with the conventional clinical diagnosis. The same analysis procedure using clinical DSM diagnoses as the criteria was best described by a single severity continuum (schizophrenia worse than schizoaffective disorder worse than bipolar psychosis); this was not the case for biotypes. These data illustrate how multiple pathways may lead to clinically similar psychosis manifestations, and they provide explanations for the marked heterogeneity observed across laboratories on the same biomarker variables when DSM diagnoses are used as the gold standard.

Biomarkers of Abnormalities of Visual Information Processing

Dynamic visual information is encoded to support the perception of movement. A variety of abnormalities have been observed in the processing of motion information in schizophrenia. Both psychotic and manic symptoms of schizophrenia are associated with visual processing abnormalities. Further investigation of the neural basis and functional consequences of this abnormal motion processing are needed in order to find a basic biomarker for assessment and intervention of cognitive dysfunction in schizophrenia (Chen 2011). Deficits in schizophrenia are detectable through psychophysical contrast and motion sensitivity, visual backward-masking, ERP (P1 and N1 visual evoked potentials) and oscillatory (signal power and phase-locking factor of evoked oscillations) measures and their validity has been tested as trait or state biomarkers of the disease (Koychev et al. 2011). These authors concluded that specific impairment confined to the magnocellular component of the visual system might be a biomarker of schizophrenia.

Genetic Biomarkers of Schizophrenia

Genomics studies have hinted towards candidate schizophrenia susceptibility chromosomal loci and genes. Current genetic research has begun to identify genes associated with schizophrenia, some of which have phenotypes that appear early in life. While these phenotypes have low predictive power for identifying individuals who

will become psychotic, they do serve as biomarkers for pathophysiological processes that can become the targets of prevention strategies. For example, alpha nicotinic receptor and its gene CHRNA7 on chromosome 15 plays a role in the neurobiology and genetic transmission of schizophrenia.

Gene Expression Analysis of Blood for Biomarkers of Schizophrenia

A study using microarrays for analysis of gene expression in blood specimens has shown that the following genes are significantly altered in schizophrenic patients: glucose transporter, SLC2A3 and actin assembly factor DAAM2 were increased, whereas translation, zinc metallopeptidase, neurolysin 1 and myosin C were significantly decreased (Kuzman et al. 2009). Expression of these candidate biomarkers was also analyzed in a longitudinal study (12–24 months) in schizophrenic patients who achieved full remission. Expression of DAAM2 returned to control levels in during remission after the first psychotic episode, suggesting that its expression correlates with diseases progression and/or response to treatment. Other studies have reported gene expression alterations in peripheral blood cells (PBC) obtained from patients with schizophrenia as compared to healthy controls (Yao et al. 2008). These alterations can be regarded as potential biomarkers. Expression levels of several genes were compared between hospitalized patients with schizophrenia and healthy controls using quantitative real-time PCR. A significant elevation was confirmed for transcripts from the gene CXCL1 but not from the other genes investigated. APOBEC3B gene expression was inversely correlated with duration of neuroleptic treatment.

Metabolic Biomarkers of Schizophrenia

NMR spectroscopy in conjunction with computerized pattern recognition analysis has been employed to investigate metabolic profiles of a total of CSF samples from drug-naive or minimally treated patients with first-onset schizophrenia and healthy controls. Short-term treatment with antipsychotic medication resulted in a normalization of the disease signature in over half the patients, well before overt clinical improvement. No normalization is observed in patients in which treatment had not been initiated at first presentation, providing molecular evidence for the importance of early intervention for psychotic disorders. Short-term treatment with atypical antipsychotic medication results in a normalization of the CSF disease signature in half the patients well before a clinical improvement would be expected. These observations suggest that the initiation of antipsychotic treatment during a first psychotic episode may influence treatment response and/or outcome.

Proteomic Studies for Biomarkers of Schizophrenia

Only a few studies on basic proteomics have been conducted for psychiatric disorders. MALDI-TOF/TOF-MS enables the discovery of proteins and biological marker fingerprinting profiling. This approach can explain the pathogenesis of neuropsychiatric disease and provide more objective testing methods. Proteomic technologies have also been applied to CSF in an attempt to discover biomarkers of schizophrenia. Although clinical proteomics in schizophrenia have yet to reveal a biomarker with diagnostic specificity, methods that better characterize the disorder using endophenotypes can advance findings. Schizophrenia biomarkers could potentially revolutionize its psychopharmacology, changing it into a more hypothesis and genomic/proteomic-driven science.

Biomarkers of Suicide

Suicides are a leading cause of death in psychiatric patients, and in the society at large; ~1 million people die of suicide worldwide each year. Suicide is a potentially preventable cause of death; therefore there is a need for development of biomarkers for predicting and tracking suicidal states. Building on their previous work on blood biomarkers of mood disorders and psychosis, investigators have examined differential expression of genes in the blood of subjects with a major mood disorder (bipolar disorder), a high-risk population prone to suicidality (Le-Niculescu et al. 2013). They used a comprehensive 'convergent functional genomics' approach to integrate genetic and functional genomics as a Bayesian strategy for reducing the false-positives and false-negatives inherent in each approach. Blood levels of SAT1 (spermidine/ spermine N1–acetyltransferase 1), the top biomarker identified at the time of testing for this study, differentiated future as well as past hospitalizations with suicidality, in a live cohort of bipolar disorder subjects, and exhibited a similar but weaker pattern in a live cohort of psychosis (schizophrenia/schizoaffective disorder) subjects. Three other (phosphatase and tensin homolog (PTEN), myristoylated alanine-rich protein kinase C substrate (MARCKS), and mitogen-activated protein kinase kinase kinase 3 (MAP3K3) showed similar but weaker effects. The authors also conducted bioinformatic analyses to identify biological pathways, mechanisms and medication targets. Overall, suicidality may be underlined, at least in part, by biological mechanisms related to stress, inflammation and apoptosis. Although this work is an important step in the search for psychiatric biomarkers, the small sample size means the results will have to be validated in much larger groups and tested for specificity and sensitivity before the results could be used clinically.

DNA methylation scan has identified an additive epigenetic and genetic association with suicide at rs7208505 within the 3′ untranslated region of the SKA2 gene

(Guintivano et al. 2014a). SKA2 gene expression was significantly lower in suicide decedents and was associated with genetic and epigenetic variation of rs7208505, possibly mediated by interaction with miR-301a. Analysis of salivary cortisol measurements suggested that SKA2 epigenetic and genetic variation may modulate cortisol suppression, consistent with its implicated role in glucocorticoid receptor transactivation. SKA2 significantly interacted with anxiety and stress to explain about 80% of suicidal behavior and progression from suicidal ideation to suicide attempt. The work is the first step in developing a blood test for identifying persons at highest risk of suicide.

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Chapter 15
Biomarkers of Cardiovascular Disorders

Epidemiology of Cardiovascular Disease

Cardiovascular disease (CVD) has remained the leading cause of death worldwide despite the tremendous progress made in medical and surgical treatment for this disease. In the US, approximately 70 million persons have symptoms or findings (without symptoms) pertaining to coronary artery disease (CAD); of these patients, 10% have clinically confirmed disease. Not all of these patients have coronary artery disease, as some may have only angina pectoris but no demonstrable pathology in the coronary arteries. On the other hand, there are patients with coronary artery disease who may not have any investigations or require hospitalization. Sometimes it is postmortem finding in patients who die of other causes. The incidence is 1 million myocardial infarctions per year and 700,000 coronary-related deaths per year in the US. Nearly 8 million Americans alive today have suffered at least one heart attack and so are at greater risk for congestive heart failure (CHF) or another, potentially fatal, heart attack. Each year, of more than 1.2 million Americans who suffer heart attacks, about 400,000 develop CHF due to damage to the heart, and half of these die within 5 years. People who have had a heart attack have a sudden death rate that is 5–6 times greater than in the general population. Hypertension (HPN) affects about 70 million persons in the US with an overlap with those suffering from CAD.

The prevalence of peripheral arterial disease (PAD) in the general population has been estimated to approach 12% in the US. About one third of patients with CAD also suffer from PAD. The most common cause of PAD is arteriosclerosis obliterans – segmental arteriosclerotic narrowing or obstruction of the lumen of the arteries supplying the limbs. The lower limbs are involved more frequently than the upper limbs. Although not life-threatening, this condition causes considerable pain and disability.

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There is overlap in the patient populations with various cardiovascular disorders. For example, hypertension is a risk factor for heart disease and can be found in patients with CAD. Most of the myocardial ischemia is due to angina pectoris but some of these patients have myocardial infarction. CHF can occur in patients with myocardial infarction.

Biomarkers of Cardiovascular Diseases

The major use of cardiac biomarkers until very recently has been the detection of myocardial infarction (MI). Although ECG is the best known classical biomarker of heart disease, the emphasis in this report is on molecular biomarkers. The rationale of using the measurement of a protein in blood for detection of MI is straightforward. The myocyte is the major cell in the heart, and the heart's purpose is to pump blood. Because myocytes essentially cannot be regenerated, if heart cells die, then cardiac function has a high probability of being impaired. When the cell dies, the proteins inside the cell will be released, with proteins in the cytoplasm leaving the cell more rapidly than ones in membranes or fixed cell elements. The most sensitive markers should be those in highest abundance in the cell, and because the major function of the heart is contraction, the proteins involved in contraction and producing the energy to support it should be good candidates for biomarkers in blood. If such proteins have cardiac-specific forms, then specificity might be achievable as well as sensitivity. Two classical biomarkers for MI were serum creatine kinase and troponin. Elevated levels of C-reactive protein (CRP) are associated with increased risks of ischemic heart disease. One of the sequelae of MI is CHF and biomarkers for this are also discussed. Systemic hypertension itself is a marker for heart disease and no separate markers are listed apart from genetic markers of hypertension. The relative risk of coronary heart disease is better predicted by two biomarkers than a single biomarker.

With advances in biotechnology, several new techniques are evolving for applications in diagnosis and therapy of cardiovascular disorders (Jain 2011). More sophisticated multiplex panels of biomarkers have emerged from work with microarrays. A classification of biomarkers for cardiovascular diseases is shown in Table 15.1. There are some overlaps of biomarkers between different categories.

Biomarkers of Acute Myocardial Infarction

Relationship of various biomarkers to pathophysiology of acute MI is shown in Fig. 15.1.

Cardiac Troponin is the biomarker of choice for diagnosing acute MI and is described in more detail later in this chapter. However, a multi-biomarker panel can improve diagnosis, risk stratification and determination of prognosis of patients

Table 15.1 Classification of biomarkers for cardiovascular diseases

Genetic biomarkers for heart disease (see Table 15.2 **for more details)**
IL-1 gene and IL-6R pathway variations
Polymorphisms in the apolipoprotein E (APOE) gene
Mutations in the low density lipoprotein (LDL) receptor gene
Mutations within several genes that code for ion channels
Polymorphism in the angiotensinogen gene promoter
SNP in the CD93 gene
Biomarkers for ischemic heart disease and acute myocardial infarction (AMI)
Acute phase reactants: C reactive protein (CRP)
Biomarkers of myocardial dysfunction: natriuretic peptide
Biomarkers of myocardial infarction: troponins, creatine kinase (muscle brain), myoglobin
Citric acid pathway metabolites
C-terminal-provasopressin (Copeptin)
Cripto-1
Fatty acid binding protein
Fetuin-A
High density lipoprotein 2 (HDL_2)
Inflammatory cytokines: IL-6 and TNF-α
Markers of ischemia: choline, ischemia-modified albumin, unbound free fatty acids
MicroRNAs: miR-208a
Plaque destabilization: intercellular adhesion molecules, vascular adhesion molecules, metalloproteinase-9
Plaque rupture: Soluble CD40 ligand, placental growth factor
Biomarkers of left ventricular hypertrophy and heart failure
Angiogenesis biomarkers of coronary heart disease with asymptomatic left ventricle dysfunction
Elevated fibroblast growth factor (FGF)-23 levels in elderly
Vitamin D deficiency in elderly
Beta-2a protein
Biomarkers of inflammation: galectin-3, CRP, TNF-α, Fas (APO-1), IL-1, IL-6, IL-18
Desmin
G protein-coupled receptor kinase-2
MMP-CV2: microparticle protein biomarker of ischemic cardiac failure
Myocyte stress: natriuretic peptide, midregional fragment of proadrenomedullin, ST-2
Neurohormones: norepinephrine, renin, angiotensin II, aldosterone, arginine vasopressin, endothelin
Oxidative stress: oxidized LDLs, myeloperoxidase, urinary biopyrrins, plasma malondialdehyde
Urinary and plasma isoprostanes
Biomarkers of risk factors for coronary heart disease
Biomarkers of inflammation: CRP and fibrinogen
Biomarkers of oxidative damage: endothelial progenitor cells (EPCs)
Plasma apolipoprotein AI and B levels

(continued)

Table 15.1 (continued)

Biomarkers for atherosclerosis
Adipocyte enhancer-binding protein 1
Asymmetric dimethylarginine (an endogenous nitric oxide synthase inhibitor)
Cathepsin D
E-selectin
Ghrelin
HSP-27 (low levels)
Lipid-modified proteins
Lipoprotein-associated phospholipase A2
Macrophages chemoattraction
Nitric oxide: impairment of production
Oxidative stress
Serum amyloid A
T-cell chemokine activity
Imaging biomarkers of cardiovascular disease
Miscellaneous: chromogranin, osteoprotegerin, adiponectin, growth differentiation factor 15, YKL-40

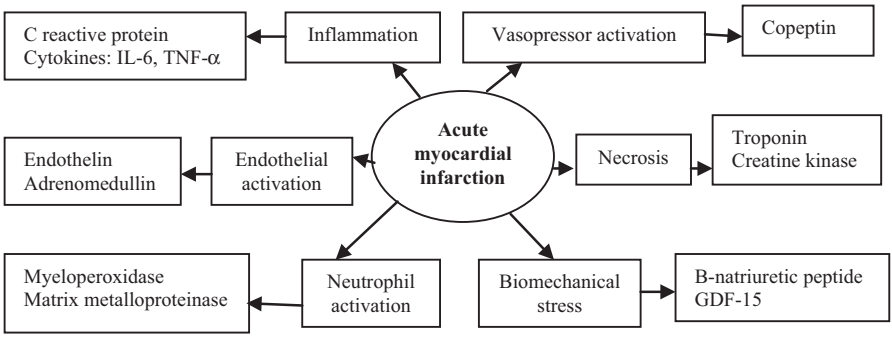

Fig. 15.1 Biomarkers of acute myocardial infarction related to pathophysiology © Jain PharmaBiotech

with acute MI. Of the various biomarkers, the ones most likely to be adopted into bedside practice in the near future are NTproBNP, MRproANP, MRproADM, copeptin and GDF-15.

Genetic Biomarkers of Cardiovascular Disorders

Several cardiovascular diseases have a genetic component, and a family history of heart disease always attracts the physician's attention. Molecular genetics has contributed to the development of molecular cardiology, opening up some new

Table 15.2 Genes that cause cardiovascular diseases

Category	Disease	Gene/mutation	Function
Atherosclerosis	Coronary artery disease	E-S128R	Monitors white blood cell adhesion to the arterial wall
	Coronary artery inflammatory disease	Interleukin-1 receptor antagonist (IL-1ra) gene	IL-1ra is a potent natural mechanism for controlling IL-1, and inflammation
Aortic-valve calcification	Precursor of aortic-valve stenosis with sequelae	SNP in LPA locus rs10455872 (Thanassoulis et al. 2013)	LPA (lipoprotein a) is atherogenic
Blood lipid disorders	Familial hypercholesterolemia	LDL	Regulation of low-density lipoprotein
	Familial dyslipoproteinemias	ApoE	Regulation of plasma lipid concentrations
Cardiac arrhythmias	Long QT syndrome	KLVQT1 HERG minK	Potassium channel
	Idiopathic ventricular fibrillation (Brugada syndrome)	SCN5A	Sodium channel
	QT-related cardiac arrhythmia with sudden death	NOS1AP	Gene is regulator of nNOS, which modulates cardiac repolarization
Cardiomyopathy	Early onset cardiomyopathy	alpha-kinase 3 (ALPK3)	Production of the protein in heart and skeletal muscles
	Familial hypertrophic cardiomyopathy	AMP-activated protein kinase β myosin Troponin T Troponin I Cardiac myosin binding protein C α tropomyosin	Muscle contraction (forced generation)
	Idiopathic dilated cardiomyopathy	Actin Dystrophin	Muscle contraction (force transduction)
Congenital malformations	Atrial septal defect	NKX2–5	Transcription factor
	Holt-Oram syndrome (holes between the atria)	TBX5	Transcription factor
Heart failure	Congestive heart failure	Wild type KIF6	Kinesin family member 6
Hypertension	Essential hypertension	AGT	Contraction of arterial smooth muscle

(continued)

Table 15.2 (continued)

Category	Disease	Gene/mutation	Function
Myocardial infarction	Early onset	VAMP8	Platelet degranulation
	Early onset	HNRPUL1	Encodes a ribonuclear protein
Right ventricular dysfunction	Pulmonary arterial hypertension	Bone morphogenetic protein receptor type-2 (BMPR2)	A receptor of the transforming growth factor-β family
Thrombotic disorders	Venous thrombosis Stroke	Factor V (Leiden mutation)	Procoagulant normally by activated protein C

pathways to the diagnosis, prevention, and treatment of some cardiovascular diseases. Genetic approaches have defined the molecular basis of an increasing array of heart diseases, such as hypertrophic cardiomyopathy and the long-QT syndrome, associated with serious arrhythmias. Knowledge of the mechanism by which single genes can cause disease, even though such mechanisms are uncommon, has led to an understanding of the pathophysiological basis of more common cardiovascular diseases, which are genetically complex. Polymorphisms in the CRP gene are associated with marked increases in CRP levels and thus with an increase in the risk of ischemic vascular disease, but these polymorphisms are not in themselves associated with the increased risk. Some of the genes that cause cardiovascular diseases are shown in Table 15.2.

A metaanalysis of genome-wide association studies of coronary artery disease (CAD) comprising 22,233 individuals followed by genotyping of top association signals in 56,682 additional individuals identified 13 loci newly associated with CAD and confirmed the association of ten previously reported CAD loci (Schunkert et al. 2011). The new loci were associated with an increase in the risk of CAD, but only three of the new loci showed significant association with traditional CAD risk factors, and the majority lie in gene regions not previously implicated in the pathogenesis of CAD. Finally, five of the new CAD risk loci have strong association with various other human diseases or traits.

Methods for Identification of Cardiovascular Biomarkers

Application of Proteomics for Biomarkers of Cardiovascular Disease

Several protein biomarkers of cardiovascular disease can be detected by conventional ELISA tests. Proteomics has opened new avenues in the search for clinically useful biomarkers of cardiovascular disease. As the number of proteins that can be

detected in plasma or serum (the primary clinical diagnostic samples) increases towards 1000, a paradoxical decline has occurred in the number of new protein biomarkers approved for diagnostic use in clinical laboratories. MicroParticle Proteomics is developing technology for detection of protein microparticle biomarkers of cardiovascular diseases. Considering the limitations of current proteomics protein discovery platforms, an alternative approach is proposed that is applicable to a range of biological/physiological problems, in which quantitative MS methods developed for analytical chemistry are employed to measure limited sets of candidate biomarkers in large sets of clinical samples. Several candidate biomarker proteins with associations to cardiovascular disease have been reported as a starting point for such a 'directed proteomics' approach.

Targeted MS-based Pipeline Approach

A targeted MS-based pipeline approach has been developed to integrate the proteomic technologies used from the discovery to the verification stages of plasma biomarker identification and applied to identify early biomarkers of cardiac injury from the blood of patients undergoing a therapeutic, planned myocardial infarction (PMI) for treatment of hypertrophic cardiomyopathy (Addona et al. 2011). The researchers used information on proteins being differentially expressed in fluid from the heart before and after this treatment to narrow in on biomarkers for cardiovascular injury in peripheral blood samples. In the process, they identified some biomarkers that also signal spontaneous heart attack. Sampling of blood directly from coronary sinus vein before, during and after controlled myocardial injury ensured enrichment for candidate biomarkers, and enabled patients to serve as their own biological controls. LC-MS/MS analyses detected 121 highly differentially expressed proteins, including previously validated biomarkers of cardiovascular disease and >100 novel candidate biomarkers for MI. Accurate inclusion mass screening (AIMS) qualified a subset of the candidates based on highly specific, targeted detection in peripheral plasma, including some biomarkers unlikely to have been identified without this step. Analyses of peripheral plasma from controls and patients with PMI or spontaneous MI by quantitative multiple reaction monitoring MS or immunoassays suggest that the candidate biomarkers may be specific to MI. During the discovery phase, MS may not detect all of the potential biomarker peptides or pull out the best possible biomarkers, but targeted methods that also incorporate information from predictive algorithms can increase the chances of finding useful candidate biomarkers. This study demonstrates that modern proteomic technologies, when coherently integrated, can yield novel cardiovascular biomarkers meriting further evaluation in large, heterogeneous cohorts. Follow-up experiments indicated that at least some of the biomarkers are found at elevated levels in blood samples from individuals experiencing spontaneous heart attacks, even several hours after the heart attack has occurred. Coupled with data from PMI patients, the pipeline strategy could find biomarkers that appear quite quickly after cardiovascular damage occurs and remain elevated after the event. This would qualify as an ideal biomarker that shows up within minutes after the event, stays up, and is detected in the peripheral blood of that

patient. The team plans to continue using this pipeline approach to assess biomarkers for heart attack as well as other cardiovascular conditions, cancer, and infectious diseases such as tuberculosis, HIV, and malaria.

Cardiovascular Disease Biomarker Panel

The future holds great promise for the availability of a panel of cardiac serum biomarkers able to delineate different stages of each heart disease, thus enabling the design of clinical interventions using stage-specific therapeutics. This is feasible only with detailed information about the unique and selective protein modifications that occur during the development of heart disease. Combination of proteomic biomarkers with clinical phenotypes and genetic haplotype information can lead to a more precise diagnosis and therapy on an individual basis for personalized medicine.

Detection of Biomarkers of Myocardial Infarction in Saliva by a Nanobiochip

The feasibility and utility of saliva as an alternative diagnostic fluid for identifying biomarkers of acute myocardial infarction (AMI) has been investigated. A lab-on-a-chip method was used to assay 21 proteins in serum and unstimulated whole saliva procured from AMI patients within 48 h of chest pain onset and from apparently healthy controls (Floriano et al. 2009). Both established and novel cardiac biomarkers demonstrated significant differences in concentrations between patients with AMI and controls. The saliva-based biomarker panel of CRP, myoglobin, and myeloperoxidase showed diagnostic capability, which was better than that of ECG alone. When used in conjunction with ECG, screening capacity for AMI was enhanced and was comparable to that of a panel of brain natriuretic peptide, troponin-I, creatine kinase-MB, and myoglobin. To translating these findings into clinical practice, the whole saliva tests were adapted to a nanobiochip platform, which may provide a convenient and rapid screening method for cardiac events at point-of-care.

Metabolomic Technologies for Biomarkers of Myocardial Ischemia

Advances in metabolic profiling technologies offer the possibility of identifying novel biomarkers and pathways activated in myocardial ischemia. Blood samples obtained before and after exercise stress testing from patients who demonstrate inducible ischemia can be used for this purpose. Plasma is fractionated by liquid

chromatography (LC), and profiling of analytes can be performed with a high-sensitivity electrospray triple-quadrupole mass spectrometer under selected reaction monitoring conditions. Lactic acid and metabolites involved in skeletal muscle AMP catabolism usually increase after exercise but there is significant discordant regulation of multiple metabolites that either increase or decrease in myocardial ischemia as compared to normal controls. Members of the citric acid pathway were among the most changed metabolites. Application of metabolomics to acute myocardial ischemia has identified novel biomarkers of ischemia, and pathway trend analysis has revealed coordinate changes in groups of functionally related metabolites.

Proton nuclear magnetic resonance (^{1}H NMR) spectra of human serum can diagnose and determine the severity of coronary artery disease. It can provide an accurate and rapid diagnosis of coronary artery disease and be used for screening as well as for effective targeting of treatments such as statins.

Imaging Biomarkers of Cardiovascular Disease

Molecular imaging techniques, MRI and PET, reveal processes at the molecular level, e.g. expression or activity of a biomarker or the activity of a biological pathway. Molecular targets that are currently being investigated experimentally by molecular imaging probes in vivo have an important role in the development of atherosclerosis and acute plaque rupture, as well as in myocardial disease (Shaw 2009). Molecular imaging technology is now being translated into clinical application in humans for diagnosis, risk assessment and response to treatment. Imaging-based surrogate biomarker end points could facilitate the development of new drugs, particularly those with novel mechanisms of action. Molecular imaging, by revealing multiple biomarkers and pathways function in vivo, will contribute to an understanding of cardiovascular disease biology at systems-level.

Annexin A5 as an Imaging Biomarker of Cardiovascular Disease

Annexin A5, a plasma protein, has strong affinity for phosphatidylserine (PS) – a plasma cell membrane phospholipid. Coupling of Annexin A5 to contrast agents enables in vivo visualization of apoptotic cell death, which is manifested by externalization of PS (Laufer et al. 2008). These imaging studies have provided novel insight into the extent and kinetics of apoptosis in cardiovascular disease. Furthermore, Annexin A5 imaging has proven to be a suitable imaging biomarker for the evaluation of apoptosis modifying compounds and plaque stabilizing strategies. Annexin A5 not only binds to exteriorized PS, but is also internalized through an Annexin A5 specific mechanism indicating that Annexin A5 imaging can also be used to visualize inflammation and cell stress. This will enable a better understanding of pathological processes underlying cardiovascular diseases.

Cardiovascular MRI

Cardiovascular MRI is a powerful tool for detecting cardiovascular biomarkers and has an important role, particularly in visualizing several reversible and irreversible myocardial tissue changes. It has potential applications in non-ischemic and inflammatory cardiomyopathies. Cardiac MRI is helpful in making a differential diagnosis between ischemic and dilated cardiomyopathy; identifying patients with myocarditis; diagnosing cardiac involvement in sarcoidosis and Chagas' disease; identifying patients with unusual forms of hypertrophic cardiomyopathy; and defining the sequelae of ablation treatment for hypertrophic obstructive cardiomyopathy.

Cardiovascular Hybrid Imaging

Cardiac hybrid imaging combines different imaging modalities in a way where both modalities equally contribute to image information. The most common and best-studied approach is to combine computed tomography coronary angiography (CTCA) and myocardial perfusion imaging either with single-photon emission computed tomography (SPECT) or with PET. This combination is a promising tool for evaluation of coronary artery disease since it enables visualization of coronary atherosclerotic lesions and their hemodynamic consequences in a single study and it appears to offer superior diagnostic accuracy when compared with stand-alone imaging (Gaemperli et al. 2012). PET-CT imaging offers superior diagnostic accuracy in patients with intermediate risk for CAD compared with stand-alone imaging. Novel, commercially available hybrid scanners containing PET and MRI as well as development of targeted probes to evaluate molecular and cellular disease mechanisms are expected to provide many new applications for cardiac hybrid imaging (Saraste and Knuuti 2012). More recent applications are a combination of CTCA and MRI by using software image fusion and utilization of commercially available hybrid PET/MRI scanners for cardiac applications. The development of new molecular imaging probes will also open completely new possibilities for guidance and monitoring of advanced therapies. An integrated Time-of-Flight (TOF) PET-MRI has advantages over current sequential imaging systems that extend beyond simple combination of molecular PET with structural MRI as it enables simultaneous view of structure and function in vivo.

Myocardial Perfusion Imaging

Myocardial perfusion imaging (MPI) is used as a primary screen to identify the presence of coronary artery disease (CAD) as evidenced by detection of areas of poor blood flow in the heart that can be caused by the formation of plaques that block the normal flow of blood to the heart. A pharmacologic stress agent is used to increase blood flow through coronary arteries temporarily during stress testing in order to more strikingly define areas of the heart that receive poor blood flow.

The adenosine A2A receptor is the receptor subtype responsible for coronary vasodilation, or the widening of blood vessels.

Stedivaze (apadenoson) is a potent agonist of the A2A and offers improved selectivity over other adenosine receptor subtypes (A1 and A2B). Phase II studies suggest that Stedivaze produces ample coronary vasodilatory activity needed for cardiac stress MPI and has a pharmacokinetic profile that will allow it to be administered as a fixed dose bolus injection. Because of its improved selectivity for the A2A receptor subtype and its optimal pharmacokinetic profile, Stedivaze may offer improved tolerability over other adenosine receptor agonists currently marketed for use in pharmacologic stress MPI. Stedivaze is in phase III clinical development by Clinical Data Inc. for use as a pharmacologic agent for MPI with the goals of demonstrating equal efficacy and improved tolerability compared to adenosine. Over 7.6 million MPI tests were performed in the US in 2008 and ~3.5 million of these tests required the use of a pharmacological agent to generate maximum coronary blood flow in lieu of exercise. With increasing aging population, there is a rise in the number of patients unable to perform exercise during diagnostic procedures, and emerging imaging modalities that require the use of a vasodilator as a biomarker.

Implantable Magnetic Biosensors for Detecting Cardiac Biomarkers

Molecular biomarkers are used as objective indicators of pathologic processes in the heart. Although their levels often change over time, their measurement is often constrained to a single time point. Cumulative biomarker exposure would provide a fundamentally different kind of measurement to what is available in the clinic. Magnetic resonance relaxometry has been used to noninvasively monitor changes in the relaxation properties of antibody-coated magnetic particles when they aggregate upon exposure to a biomarker of interest (Ling et al. 2011). The authors used implantable devices containing such sensors to continuously profile changes in three clinically relevant cardiac biomarkers at physiological levels for up to 72 h. Sensor response differed between experimental and control groups in a mouse model of myocardial infarction and correlated with infarct size. The prototype for a biomarker monitoring device also detected doxorubicin-induced cardiotoxicity and can be adapted to detect other molecular biomarkers with a sensitivity as low as the pg/ml range.

Applications of Biomarkers of Cardiovascular Disease

Novel biomarkers have improved diagnosis and prediction of outcome in AMI, but none of the new prognostic biomarkers have been tested and proven to alter outcome of therapeutic intervention. Randomized trials are urgently needed to address

this translational gap before the use of novel biomarkers becomes common practice to facilitate personalized management following an acute coronary event.

Biomarkers for Ischemic Heart Disease and Myocardial Infarction

Evaluation of patients who present to the hospital with a complaint of chest pain or other signs or symptoms suggestive of acute coronary syndrome is time-consuming, expensive, and problematic. Recent investigations have indicated that increases in biomarkers upstream from biomarkers of necrosis (cardiac troponins I and T), such as inflammatory cytokines, cellular adhesion molecules, acute-phase reactants, plaque destabilization and rupture biomarkers, biomarkers of ischemia, and biomarkers of myocardial stretch may provide earlier assessment of overall patient risk and aid in identifying patients with higher risk of an adverse event. Only two categories of the biomarkers are approved – troponins and natriuretic peptide. Specifications that have been addressed for these biomarkers will need to be addressed with the same scrutiny for the newer biomarkers under investigation. These include validating analytical imprecision and detection limits, calibrator characterization, assay specificity and standardization, pre-analytical issues, and appropriate reference interval studies. Crossing boundaries from research to clinical application will require replication in multiple settings and experimental evidence supporting a pathophysiologic role and, ideally, interventional trials demonstrating that monitoring single or multiple biomarkers improves outcomes.

Troponin

The contractile unit (or sarcomere) of striated muscle fiber is composed of thick and thin filaments. The thick filament is composed mainly of myosin. Actin, tropomyosin, and troponin comprise the thin filament. Muscle contraction occurs when the thick and thin filaments slide past each other, thereby shortening the length of the sarcomere. The interaction between the thick and thin filaments is regulated by the troponin complex found on the thin filaments. The troponin complex is composed of three protein subunits: troponin-I (TnI), troponin-T (TnT), and troponin-C (TnC). The calcium-mediated contraction of striated muscle (fast-skeletal, slow-skeletal, and cardiac muscle) is regulated by the troponin complex; contraction of smooth muscle is regulated by calmodulin. Three distinct tissue-specific isoforms of TnI have been identified: two in skeletal muscle and one in cardiac muscle, cTnI, which has never been isolated from skeletal muscle. Within the heart, cTnI appears to be uniformly distributed throughout the atria and ventricles. This absolute specificity of cTnI for cardiac tissue makes it an ideal biomarker of myocardial injury. It is released into the circulation when myocardial injury occurs and has become the

basis for a standard test in combination with clinical and electrocardiographic findings for physicians to conduct prompt and effective triage of patients presenting with chest pain. Since the FDA first cleared an assay for cardiac troponin testing in 1996, the number of cTnI and cTnT assays have increased to >20, in both quantitative and qualitative formats, using both central laboratory and POC testing.

A chemiluminescent microparticle immunoassay for the quantitative determination of cardiac troponin I in human serum and plasma on an automated immunoassay instrument system (ARCHITECT) can be used to aid in the diagnosis of MI. Cardiac troponin POC testing using i-STAT cTnI assay is also useful for risk for predicting adverse outcomes in acute coronary syndrome patients.

Cardiac troponin monitoring for detection of myocardial injury has been designated the new standard for differentiating the diagnosis of unstable angina and non-ST-elevation (STEMI) in acute coronary syndrome patients. Increased cardiac troponin I (cTnI) or T (cTnT) in the clinical setting of ischemia is defined as an acute MI and has been endorsed by the European Society of Cardiology, American College of Cardiology, and the American Heart Association. The clinical significance of increased concentrations of cardiac troponins observed in patients with renal failure in the absence of acute coronary syndrome is controversial. Troponin observed in serum of renal failure patients is predominantly the free intact form, as in patients with acute coronary syndrome and is considered to reflect cardiac pathology.

A multicenter study tested the diagnostic accuracy of four sensitive cardiac troponin assays (Abbott's Architect Troponin I, Roche High-Sensitive Troponin T, Roche Troponin I, and Siemens' Troponin I Ultra) as well as a standard assay (Roche Troponin T) that were performed on blood samples obtained in the emergency department from patients who presented with symptoms suggestive of acute MI (Reichlin et al. 2009). The diagnostic performance of sensitive cardiac troponin assays was excellent, and these assays can substantially improve the early diagnosis of acute MI, particularly in patients with a recent onset of chest pain. Another multicenter study determined levels of troponin I as assessed by High-Sensitive Troponin T and traditional biomarkers of myocardial necrosis in patients with suspected acute MI, on admission and 3 h and 6 h after admission (Keller et al. 2009). It concluded that the use of a sensitive assay for troponin I improves early diagnosis of acute MI and risk stratification, regardless of the time of onset of chest-pain.

In most patients with stable coronary artery disease, plasma cardiac troponin T levels are below the limit of detection for the conventional assay. A study with follow-up period of >5 years used a new, high-sensitivity assay to determine the concentration of cardiac troponin T in plasma samples from patients with stable coronary artery disease and analyzed the results of the assay in relation to the incidence of cardiovascular events (Omland et al. 2009). After adjustment for other independent prognostic indicators, cardiac troponin T concentrations as measured with a highly sensitive assay were significantly associated with the incidence of cardiovascular death and heart failure but not with MI in patients with stable coronary artery disease.

High-sensitive TnC assays have two differentiating features from contemporary TnC assays: (1) detection of TnC in healthy persons; and (2) a precise definition of what is "normal". Recent multicentre studies have shown that high-sensitive TnC assays improve the early diagnosis of AMI. To achieve the best clinical use, TnC has to be interpreted as a quantitative variable. Rising and/or falling levels differentiate acute from chronic cardiomyocyte necrosis. "Detectable" levels will become the norm and have to be clearly differentiated from "elevated" levels. The differential diagnosis of a small amount of cardiomyocyte necrosis and therefore mild elevation of TnC is broad and includes acute and chronic cardiac disorders. The differential diagnosis of a large amount of cardiomyocyte necrosis and therefore substantial elevation of TnC is much smaller and largely restricted to AMI and myocarditis (Twerenbold et al. 2011).

Cardiac biomarker (preferably cardiac troponin) level > 3 times the upper reference limit is indicative of a periprocedural (coronary angioplasty and stenting) MI. At the higher estimate, the incidence of these events is similar to the annual rate of major spontaneous MI. Mechanism of this complication with role of biomarkers and methods for prevention as well as management have been reviewed elsewhere (Prasad and Herrmann 2011).

Cardiac troponin concentrations have been used to identify patients who would benefit from urgent revascularization for acute coronary syndromes. However, a study on large number of patients has shown that cardiac troponin T concentration was an independent predictor of death from cardiovascular causes, MI, or stroke in patients who had both type 2 diabetes and stable ischemic heart disease. An abnormal troponin T value of 14 ng/L or higher did not identify a subgroup of patients who benefited from random assignment to prompt coronary revascularization (Everett et al. 2015).

Natriuretic Peptide

Several terms relevant to natriuretic peptide (NP) are as defined as follows:

- B-Type Natriuretic Peptide (BNP) is a biologically active hormone, which corresponds to the C terminal fragment of proBNP (amino acids 77–108) and is present in both myocardium and plasma.
- Pre-proBNP is the cellular precursor synthesized in the myocardial cell. It contains 134 amino acids, including a signal peptide of 26 amino acids and is present only in myocardial tissue.
- ProBNP contains 108 amino acids (1–108) and is produced from pre-proBNP by cleavage of the signal peptide, when appropriate signals for hormone release are given. It is present in both myocardium and plasma.
- NT-proBNP, the entire N-terminal fragment of proBNP (amino acids 1–76), lacks hormonal activity and is present in both myocardium and plasma. Further degradation products of this molecule are sometimes identified with the same abbreviation, e.g., NTproBNP (amino acids 1–21), but little metabolic and pathophysiologic information is available for these molecules.

The NP system is primarily an endocrine system that maintains fluid and pressure homeostasis by modulating cardiac and renal function. The physiologic functions of the NP system in healthy humans and in patients with cardiovascular disease are not fully understood. NP levels are elevated in patients with CHF and other cardiac diseases; measurement of NPs may be used in the clinical setting to aid diagnosis and prognosis. In addition, synthetic NPs such as nesiritide are available for use in management of patients with acutely decompensated CHF. Not only do NPs modulate volume and pressure homeostasis, but they also exert important anti-proliferative, anti-fibrotic effects in the heart. Thus, NPs may prove useful for prevention of remodeling after myocardial infarction and in advanced CHF. BNP is emerging as an important biomarker in patients with CHF and other cardiovascular diseases, such as pulmonary hypertension and atherosclerotic vascular disease. Elevated NP levels may serve as an early warning system to help to identify patients at high risk for cardiac events. Recombinant human ANP (carperitide) and BNP (nesiritide) are useful for management of acutely decompensated HF; these drugs are also being investigated for myocardial and renal protection in the setting of cardiac surgery and for prevention of cardiac remodeling. The clinical application of NPs is expanding rapidly. Basic science and clinical research findings continue to improve our understanding of the NP system and guide use of ANP and BNP as biomarkers and as therapeutic agents.

Because BNP and NT-proBNP are released mainly from the cardiac ventricles in response to increased stretch and wall tension, it is not surprising that increased plasma concentrations of these NPs have been described in CHF, asymptomatic left ventricular dysfunction, arterial and pulmonary hypertension, cardiac hypertrophy, valvular heart disease, arrhythmia, and acute coronary syndrome. Because BNP and NT-proBNP are increased in a variety of cardiac and noncardiac diseases, caution must be exercised in interpreting the results in the context of clinical situation. BNP and NT-proBNP may become components of a panel of biomarkers, along with cardiac troponin, to be used for risk stratification in acute coronary syndrome. Commercially available BNP/NT-proBNP assays are:

- Access BNP (Beckman Coulter) for diagnosis and assessing severity of HF.
- AxSYM (Abbott) for diagnosis of HF.
- Centaur BNP (Siemens/Bayer) aids in diagnosis and severity assessment of HF.
- Dimension VISTA (Siemens/Dade Behring), pending FDA clearance, to aid in diagnosis and severity assessment of HF.
- ST AIA-PACK BNP (Tosoh) is approved in Japan for diagnosis of HF with plan to launch in Europe, but not cleared by FDA.
- StatusFirst™ CHF (congestive heart failure) NT-proBNP (Nanogen, licensed from Roche) is CE-marked and has been cleared by the FDA for diagnostic use with EDTA plasma samples.
- Triage BNP (Alere), cleared by the FDA, for diagnosis and risk stratification of patients with HF.

In 2015, the FDA approval of Roche's ENTRESTO™ (sacubitril/valsartan) tablets to reduce the risk of cardiovascular death and hospitalization for heart failure in

patients with CHF (NYHA Class II-IV) and reduced ejection fraction, could shift the paradigm in the way heart failure patients will be managed using NT-proBNP in lieu of BNP for patients receiving this drug. Advantages of NT-proBNP over NT include longer half-life and stability at room temperature.

Copeptin

Copeptin (C-terminal part of the vasopressin prohormone) is secreted stoichiometrically with vasopressin. The vasopressin system is activated after acute myocardial infarction (MI). Copeptin may predict adverse outcome, especially in those with an elevated NTproBNP. Copeptin is also a biomarker for prognosis of intracerebral hemorrhage. A commercial assay for C-Terminal Provasopressin is available from Thermo Fisher. In combination with a troponin biomarker test, the copeptin assay enables physicians to rule out or confirm the onset of myocardial infarction within minutes. Elevated plasma copeptin in HF has been associated with adverse outcomes such as increased mortality, risk of hospitalization and correlates with the severity of HF. Copeptin may add prognostic information to already established predictors such as clinical variables and natriuretic peptides in HF. In addition, copeptin has been found to be a superior biomarker when compared with BNP and NT-proBNP in HF patients discharged after hospitalization caused by HF or MI. The optimal use of copeptin in HF remains unresolved and future randomized trials must determine the role of copeptin in HF as a biomarker of adverse outcomes, risk stratification or as a target in biomarker-guided therapy with arginine vasopressin-antagonists in individualized patient treatment and everyday clinical practice (Balling and Gustafsson 2014).

Creatine Kinase Muscle Brain

Creatine kinase muscle brain (CK-MB), an enzyme that is involved in muscle metabolism, is found in both heart muscle tissue and skeletal muscle, albeit at a much lower concentration in the latter. CK-MB is released into the blood stream when cardiac muscle is damaged, hits a plateau where its levels are highest, and then eventually returns to lower levels. Although CK-MB analysis is currently the benchmark for biomarker detection of MI, similar patterns of CK-MB release can be caused by renal kidney failure, skeletal muscle trauma, and other unrelated ailments.

miRNAs as Biomarkers of Acute Coronary Syndrome

Circulating miRNAs may have diagnostic potential in acute coronary syndrome (ACS). miR-208a is involved in the regulation of the myosin heavy chain isoform switch during development and in pathophysiological conditions such as

cardiac arrhythmias, cardiac remodeling, and regulation of the expression of hypertrophy pathway components (Oliveira-Carvalho et al. 2013). To assess the diagnostic and prognostic value of cardiomyocyte-enriched miRNAs in the context of clinical variables and a sensitive myonecrosis biomarker in a larger ACS cohort, miR-1, miR-133a, miR-133b, miR-208a, miR-208b, and miR-499 concentrations were measured by RT-qPCR in plasma samples obtained on admission from a large series of patients with ACS, who were followed for 6 months (Widera et al. 2011). miRNA levels were independently associated with hsTnT levels. Patients with myocardial infarction (MI) presented with higher levels of miR-1, miR-133a, and miR-208b compared with patients with unstable angina, but all 6 investigated miRNAs showed a large overlap between patients with unstable angina or myocardial infarction. MiR-133a and miR-208b levels were significantly associated with the risk of death in univariate and age- and gender-adjusted analyses. Both miRNAs lost their independent association with outcome upon further adjustment for hsTnT. This study cautions about the potential usefulness of cardiomyocyte-enriched miRNAs as diagnostic or prognostic biomarkers in ACS.

Myoglobin

Myoglobin is a heme protein found in the cytosol of both cardiac and skeletal muscle. Following the death of cardiac tissue, myoglobin is the first cardiac marker to increase above normal levels in the blood. Myoglobin measurements have proven useful as an early marker for heart attack but other non-cardiac related trauma can also cause circulating levels of myoglobin to increase, so it cannot be used exclusively.

Fatty Acid Binding Protein

Recent data suggest that serum free fatty acid concentrations increase well before markers of cardiac necrosis and are sensitive indicators of ischemia in myocardial infarction (MI). Fatty acid binding protein (FABP) is abundant in cardiac muscle and is presumed to be involved in myocardial lipid homeostasis. Similar to myoglobin, plasma FABP increases within 3 h after onet of MI and returns to reference values within 12–24 h. Heart type FAB (H-FABP) assay was approved in Japan for early diagnosis of acute MI as compared with the sensitive troponin assay. However, an observational study for measurement of H-FABP in patients with chest pain suggestive of acute MI showed no improvement in diagnosis of early acute MI as compared with the current sensitive troponin assay because of its extremely low specificity (Kagawa et al. 2013).

Growth Differentiation Factor-15

Growth Differentiation Factor-15 (GDF-15) is a member of the TGF-β cytokine superfamily. It is not normally expressed in the heart, but under episodes of ischemia and reperfusion) its levels go up in a variety of tissues, including cardiomyocytes. The level of GDF-15 rapidly increases and remains elevated in the early period of acute MI and is related to biomarkers, such as WBC, LDH, CK-MB, and TnT (Zheng et al. 2011). GDF-15 also provides prognostic information following an MI.

High Density Lipoprotein 2

Alterations in protein composition and oxidative damage of high density lipoprotein (HDL) impair the cardioprotective properties of HDL. HDL_2 of subjects with coronary artery disease (CAD) carries a distinct protein cargo and protein oxidation helps to generate dysfunctional HDL. Targeted tandem MS analysis of the model's significant features has revealed that HDL_2 of CAD subjects contained oxidized methionine residues of apolipoprotein A-I and elevated levels of apolipoprotein C-III. A proteomic signature composed of MALDI-MS signals from apoA-I, apoC-III, Lpa and apoC-I accurately differentiated between CAD and control subjects (Vaisar et al. 2010). Thus models based on selected identified peptides in MALDI-TOF MS of the HDL have diagnostic potential.

Cripto-1 as a Biomarker of Myocardial Infarction

Cripto-1, a member of the (EGF-related proteins), is implicated in carcinogenesis and is also a biomarker for infarcted cardiac tissues. It is overexpressed in infarcted myocardial tissue, and not expressed or weakly expressed in non-infarct related heart disease tissues and normal tissues. Furthermore, the overexpression of Cripto-1 correlates with the hypoxia-inducible factor-1-alpha indicating specificity to ischemic heart tissue. The expression of Cripto-1 has also been shown to be highly expressed in stem cells, which may have an important role in the repair of damaged myocardial tissue. This could represent a new biomarker for the diagnosis of myocardial infarction as well as a surrogate biomarker to monitor the healing process including regenerative stem cell activity of the infarcted myocardial tissue.

Cataract as a Biomarker of Ischemic Heart Disease

There is an association between aging of the lens and ischemic heart disease (IHD), which may well be a causative one. Both lens proteins, plasma proteins, and the collagen of the vascular walls are subject to denaturation by a spontaneous reaction between aminogroups and reducing sugars, glycotoxins from tobacco smoke, or

lipid peroxidation products. The spontaneous reaction leads to formation of advanced glycation end products (AGEs), which have been shown to increase atherogenicity of LDL-particles as well as playing a role in stiffening of arterial vessel walls both of which are important features in the pathogenesis of IHD. In the lens of the human eye, AGEs lead to accumulation of yellow, fluorescent compounds] thus causing the intrinsic fluorescence.

Cataract is a biomarker of the risk of and/or presence of cardiovascular disease, but the grading of cataract and its subclinical precursors is generally subjective and crude. Assessment of age-related changes in the lens can be used as a biomarker for IHD. Lens aging is estimated by fluorophotometric assessment in undilated eye using a non-invasive ocular fluorophotometer. Presence and severity of lens opacities can also be determined from retroilluminated lens photographs focused on the anterior lens surface taken after dilation of the pupil using a Canon 60UVi camera. The degree of cortical lens opacities can be graded and used as an indicator of tissue-damage caused by advanced glycation end products to give an estimate of the risk of IHD related to the effect of advanced glycation end products.

Plasma CD93 as a Biomarker for Coronary Artery Disease

A common SNP in the CD93 gene has been associated with risk of coronary artery disease (CAD). CD93 is a transmembrane glycoprotein, which is detectable in soluble form in human plasma. The results of a case-control study of premature MI and a nested case-control analysis of a longitudinal cohort study of 60-year-old subjects show that increased concentration of soluble CD93 in plasma is a biomarker for CAD, including MI (Mälarstig et al. 2011).

Plasma Fetuin-A Levels and the Risk of Myocardial Infarction

Fetuin-A, a protein almost exclusively secreted by the liver, induces insulin resistance and subclinical inflammation in rodents. Circulating fetuin-A levels are elevated in humans with metabolic syndrome and insulin resistance, conditions that are associated with increased risk of cardiovascular disease. A case-cohort study based on the European Prospective Investigation into Cancer and Nutrition-Potsdam Study has shown a link between high plasma fetuin-A levels and an increased risk of MI (Weikert et al. 2008).

YKL-40 as an Inflammatory Biomarker in Ischemic Heart Disease

Several inflammatory cytokines are involved in vascular inflammation resulting in endothelial dysfunction which is the earliest event in the atherosclerotic process leading to manifest cardiovascular disease. YKL-40 is an inflammatory glycoprotein involved in endothelial dysfunction by promoting chemotaxis, cell attachment

and migration, reorganization and tissue remodeling as a response to endothelial damage. YKL-40 protein expression is seen in macrophages and smooth muscle cells in atherosclerotic plaques with the highest expression seen in macrophages in the early lesion of atherosclerosis (Rathcke and Vestergaard 2009). YKL-40 has been found elevated in patients with both acute and stable chronic cardiovascular diseases and could be a useful biomarker of disease severity, prognosis and survival in patients with ischemic heart disease (Kastrup 2012). YKL-40 could be used for monitoring the therapies and for prognostic evaluations in patients with cardiovascular disease.

Biomarkers of Cardiomyopathy

Cardiomyopathy is the measurable deterioration of the myocardium to contract. Although it may apply to almost any disease affecting the heart, it is usually reserved for severe myocardial disease leading to heart failure. Biomarkers of heart failure are genetic biomarkers of inherited hypertrophicardiomyopathy are described in following sections but some special types of cardiomyopathy are included in this section.

miRNA Biomarkers of Peripartum Cardiomyopathy

Peripartum cardiomyopathy (PPCM) is a life-threatening pregnancy-associated cardiomyopathy in previously healthy women. Although PPCM is driven in part by the 16-kDa N-terminal prolactin fragment (16 K PRL), the underlying molecular mechanisms are poorly understood. A study found that 16 K PRL induced miRNA-146a (miR-146a) expression in ECs, which attenuated angiogenesis through downregulation of NRAS; 16 K PRL stimulated the release of miR-146a–loaded exosomes from ECs (Halkein et al. 2013). The exosomes were absorbed by cardiomyocytes, increasing miR-146a levels, which resulted in a subsequent decrease in metabolic activity and decreased expression of Erbb4, Notch1, and Irak1. Mice with cardiomyocyte-restricted Stat3 knockout (CKO mice) exhibited a PPCM-like phenotype and displayed increased cardiac miR-146a expression with coincident downregulation of Erbb4, Nras, Notch1, and Irak1. Blocking miR-146a with locked nucleic acids or antago-miRs attenuated PPCM in CKO mice without interrupting full-length prolactin signaling, as indicated by normal nursing activities. Finally, miR-146a was elevated in the plasma and hearts of PPCM patients, but not in patients with dilated cardiomyopathy. These results demonstrate that miR-146a is a downstream-mediator of 16 K PRL that could potentially serve as a biomarker and therapeutic target for PPCM.

Takotsubo Cardiomyopathy

Takotsubo cardiomyopathy (TC) mimics acute myocardial infarction (AMI). It has been postulated that ventricular dysfunction in TC in the absence of significant myocardial necrosis would produce higher BNP)/TnT and BNP/creatine kinase MB fraction (CKMB) ratios than in AMI. In a study on TC and 97 AMI patients, the ratios of BNP/TnT and BNP/CKMB were calculated with the use of first simultaneously drawn laboratory values (Randhawa et al. 2014). TC could be distinguished from AMI with 95% specificity with the use of BNP/TnT and BNP/CKMB ratios. The value of BNP was significantly higher in TC than in AMI. Thus early BNP/TnT and BNP/CKMB ratios help to differentiate TC from AMI with greater accuracy than BNP alone.

Troponin T Levels in Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is the most common genetic cardiovascular disorder, affecting ~1 in 500 people in the general population. It is defined as left ventricular hypertrophy in the absence of another cardiac or systemic disease such as arterial hypertension, aortic stenosis, and metabolic cardiomyopathy, which are capable of producing the magnitude of evident hypertrophy. The natural course of HCM varies considerably. Some affected patients remain completely asymptomatic throughout life, whereas others develop progressive heart failure early in life. Apart from early onset heart failure, premature sudden cardiac death (SCD) is the most feared complication. Risk stratification for SCD is imperfect and there is need for biomarkers of risk.

A study has evaluated the association of elevated high-sensitivity cardiac troponin T levels (hs-cTnT) with the severity of disease expression and adverse events in patients with HCM (Hasler et al. 2016). Results showed that elevated hs-cTnT levels in HCM patients are associated with disease severity and, potentially, with more adverse cardiac events. However, risk assessment in patients with HCM requires a multimodal approach that includes echocardiography, exercise stress testing, Holter ECG, and MRI. Biomarkers should be an integrative part of the routine work-up in HCM, prompting closer follow-up of patients with elevated hs-cTnT levels. Future studies should test whether integration of hs-cTnT in clinical decision algorithms will improve risk stratification.

Biomarkers of Heart Failure

HF, which occurs when an impaired heart muscle cannot pump blood efficiently, is a growing health problem and major cause of cardiac death. The diagnosis of heart failure may be challenging because its symptoms, e.g. dypnoea, can overlap those of other conditions. Missing a heart failure diagnosis can put patients at high risk of

serious problems, including death, but overdiagnosis may lead patients to receive unnecessary treatment. Several biomarkers of HF overlap with those of other cardiovascular disorders as several pathologies are in operation when the heart fails. Some of these biomarkers have already been discussed under other disease categories.

Annexin A5 for Prognosis of Heart Failure

Annexin A5 (anxA5) expression is increased in organs affected by HF and circulating anxA5 levels are expected to predict mortality in HF patients. Diagnostic value of anxA5 as well as NT-proBNP, CRP and estimated glomerular filtration rate (eGFR) to predict mortality in HF patients was determined in a prospective study with a median follow-up of 3.6 years; underlying mechanisms were investigated in anxA5−/− mice (Schurgers et al. 2016). AnxA5 levels were significantly elevated in HF patients compared to healthy control subjects. AnxA5, NT-proBNP and eGFR all predict mortality independently, but anxA5 significantly improved the diagnostic efficiency of NT-proBNP alone. Whereas natriuretic peptides originate from the myocardium, high circulating anxA5 levels in patients with HF are likely to reflect peripheral organ damage secondary to HF.

Angiogenesis Biomarkers

Patients with coronary artery disease (CAD) and left ventricular systolic dysfunction (LVSD) are often asymptomatic. Angiogenesis is implicated in the physiology of vascular repair and cardiac remodeling, and is one of many pathophysiological processes implicated in heart failure (HF). Plasma indices associated with angiogenesis – angiogenin, VEGF, and angiopoietin (Ang)-1 and Ang-2 – are hypothesized to be abnormal in CAD patients with LVSD, being correlated with EF and wall motion abnormalities wall motion score independently of underlying coronary atheroma score. However, study results show that levels of angiogenin are inversely related with EF and positively with coronary atheroma scores, but not independently of EF (Patel et al. 2009). Other angiogenic markers were unrelated to objective measures of LVSD but VEGF were lower amongst those patients with heart failure. Angiogenin levels were related to wall motion scores. Thus, heart failure has only a modest impact on biomarkers of angiogenesis, in patients with CAD. Further research is warranted into the diagnostic and prognostic utility of biomarkers of angiogenesis for detection of asymptomatic ventricular dysfunction.

β-2a Protein as a Biomarker of Heart Failure

Increased activity of single ventricular L-type Ca^{2+} channels is a hallmark in human HF. Ca^{2+} plays a key role in controlling heart beat and this Ca ion channel is regulated by a group of accessory proteins, a major component of which is the β-2a regulatory protein. In many types of terminal human HF, β-2a protein is increased

or over-expressed, and the electrical activity in the heart's cells show a distinct pattern of single-channel activity, an indicator that the influx of Ca into the heart has also increased. Studies in animal models of human HF process show the potential value of β-2a as a biomarker of HF and support the therapeutic possibility to lower β-2a in a heart that's beginning to fail.

Desmin

Desmin is one of the fundamental cytoskeleton proteins of cardiomyocytes, and has a mechanical, structural and regulatory function. In comparison with healthy individuals, patients with HF) present different expression of desmin content, that can be associated with its abnormal structure, different distribution, localization and disturbed function. Abnormal expression of desmin in cardiomyocytes plays a key role in progression of HF. Desmin content in cardiomyocytes obtained by endomyocardial biopsy correlates with long-term prognosis in HF patients (Pawlak et al. 2009). Low expression of desmin in cardiomyocytes with IHC assay is associated with unfavorable clinical course. Desmin is a tissue biomarkers and requires an invasive procedure for biopsy of heart muscle. Desmin is also linked to other diseases such as Alzheimer's disease and cancer; it is being investigated as a biomarker of colorectal cancer.

Galectin-3 as Biomarker of Acute Heart Failure

Galectin-3, a protein involved in inflammation and fibrosis, has been identified as biomarker for HF with the potential of further improving the challenging task of diagnosing and predicting outcomes for patients with symptoms of HF, primarily shortness of breath. Elevated blood levels of galectin-3 can help diagnose HF and identify patients at risk of dying. Combination of galectin-3 with NT-proBNP is the best predictor for prognosis in subjects with acute HF. The strong predictive power of galectin-3 indicates that HF is also an inflammatory process. The role of galectin-3 in HF may lead to new standards for therapeutic decision making or development of new agents that would inhibit this inflammatory cascade. A study on 7968 subjects from the Prevention of REnal and Vascular ENd-stage Disease (PREVEND) cohort with a median follow-up of 10 years, galectin-3 is associated with age and risk factors of CV disease (de Boer et al. 2012).

Galectin-3 levels were measured in 1996–1998 as part of a routine examination of 3353 participants enrolled in the Offspring Cohort of the Framingham Heart Study. At the time of measurement the average age of the participants was 59 years and during an average follow-up of 11 years, 166 participants (5.1%) had a first HF event (Ho et al. 2012). Among the 25% of persons with the highest galectin-3 levels (15.4–52.1 ng/ml) the annual rate of HF was 12 per 1000 people compared with 3 per 1000 people for the 25% of participants with the lowest galectin-3 levels (3.9–12 ng/ml). It was concluded that higher concentration of galectin-3 is associated with increased risk for incident HF and mortality. Future studies evaluating the role

of galectin-3 in cardiac remodeling may provide further insights into the role of Gal-3 in the pathophysiology of HF.

G Protein-coupled Receptor Kinase-2 as Biomarker of CHF

The enzyme GPCR kinase-2 (GRK2 or β-ARK1) regulates β-adrenergic receptors (β-ARs) in the heart, and its cardiac expression is elevated in HF. It is a potential biomarker for HF. Decreasing or inhibiting the enzyme reverses heart failure in animal experiments. GRK activity participates with β-AR density to regulate catecholamine-sensitive cAMP responses. Myocardial GRK2 expression and activity are mirrored by lymphocyte levels of this kinase, and its elevation in HF is associated with the loss of β-AR responsiveness and appears to increase with disease severity. Therefore, lymphocytes may provide a surrogate for monitoring cardiac GRK2 in human HF. A retrospective study has confirmed that lymphocyte GRK2 protein levels can independently predict prognosis in patients with HF (Rengo et al. 2016). There are plans to perform large human trials to specifically look at levels of GRK2 to see if they can predict responses to drugs such as β-blockers or other treatments for HF. It will also help to determine if GRK2 is a biomarker that can predict response to various therapies. If a drug lowers GRK2 levels, it should help the patient with HF. In HF, the β-adrenergic receptor system fails to work properly. One of the functions of GRK2 is to turn off β-ARs.

GRK2 has now been shown to lead to permanent damage after myocardial infarction. Over production of GRK2 following a heart attack actually stimulates pro-death pathways in myocyctes outside of the initial zone of damage. There is an inverse link between GRK2 activity and the production of NO, a molecular messenger that protects the heart against damage caused by a sudden loss of blood. When there is more GRK2, there is less NO, and vice versa. GRK2 may be affecting NO production by inhibiting the prosurvival protein kinase Akt, which itself regulates NO. These conclusions are based on a study that used gene therapy to inhibit GRK2, and found heart muscles cells in mice were substantially protected against destruction that would otherwise occur after an induced myocardial infarction (Brinks et al. 2010). Conversely, mice engineered to express excess GRK2 had more damage than would have been expected after myocardial infarction. These findings suggest that humans experiencing a heart attack might be helped with prompt delivery of a therapeutic targeting inhibition of GRK2. While it may be years before this concept can be tested in patients experiencing myocardial infarction, anti-GRK2 gene therapy could be tested in patients with HF much sooner.

KIF6 Gene as Biomarker of Heart Failure

Carriers of the KIF6 (kinesin family member 6) wild-type gene are 50–55% more likely to develop HF. KIF6 as a biomarker of CHF is the basis of a genetic test, StatinCheck, developed by Celera and offered through its subsidiary Berkeley

HeartLab. Physicians can use the KIF6 test to identify the increased risk for HF and begin treating their patients with statins. A study investigated whether 35 genetic polymorphisms, previously found to be associated with cardiovascular disease, were associated with MI in the CARE (Cholesterol and Recurrent Events) trial and CHD in the WOSCOPS (West of Scotland Coronary Prevention Study). In both the CARE and the WOSCOPS trials, carriers of the KIF6 719Arg allele had an increased risk of coronary events, and pravastatin treatment substantially reduced that risk (Iakoubova et al. 2008). Carriers of the 719Arg allele of KIF6 have 34% higher risk of MI and 24% higher risk of HD compared with noncarriers among 25,283 women from the Women's Health Study, confirming and extending previous reports (Shiffman et al. 2008).

Metabolic Biomarkers of Heart Failure

There are disturbances of several metabolites in heart failure. A high-throughput approach was applied to identify metabolic signatures and plasma diagnostic biomarkers underlying HF by 1H–NMR spectroscopy (Wang et al. 2013). The study concluded that NMR-based metabolomics approach performs well to identify diagnostic plasma biomarkers and provided new insights into metabolic process related to HF.

Trimethylamine-N-oxide (TMAO), a microbiota-dependent metabolite from dietary phosphatidylcholine and carnitine, is a strong predictor of CAD. A prospective, observational study on patients has shown that plasma levels of TMAO, choline and betaine are elevated in those with chronic HF compared to control subjects, with the highest levels in patients with NYHA classes III and IV (Trøseid et al. 2015). Furthermore, TMAO levels were highest in individuals with ischemic HF, followed by those with stable CAD and non-ischemic HF. TMAO, but not choline or betaine, was associated with reduced transplant-free survival: ~50% of patients in the upper tertile of TMAO levels died or received a heart transplant during 5.2 years of follow-up. Thus TMAO levels would provide prognostic information about adverse outcomes in chronic HF.

miRNA Biomarkers of Heart Failure

A study has determined myocardial and circulating miRNA abundance and its changes in patients with stable and end-stage HF before and at different time points after mechanical unloading by a left ventricular assist device (LVAD) by small RNA sequencing (Akat et al. 2014). miRNA changes in failing heart tissues partially resembled that of fetal myocardium. Consistent with prototypical miRNA–target-mRNA interactions, target mRNA levels were negatively correlated with changes in abundance for highly expressed miRNAs in HF and fetal hearts. The circulating small RNA profile was dominated by miRNAs, and fragments of tRNAs and small cytoplasmic RNAs. Heart- and muscle-specific circulating miRNAs (myomirs)

increased up to 140-fold in advanced HF, which coincided with a similar increase in cardiac troponin I (cTnI) protein, the established biomarker for heart injury. These extracellular changes nearly completely reversed 3 mo following initiation of LVAD support. In stable HF, circulating miRNAs showed less than 5-fold differences compared with normal, and myomir and cTnI levels were only captured near the detection limit. These findings provide the underpinning for miRNA-based therapies and emphasize the usefulness of circulating miRNAs as biomarkers for heart injury performing similar to established diagnostic protein biomarkers.

Natriuretic Peptide as Biomarker of Heart Failure

The PRIDE study showed NT-proBNP to be highly sensitive and specific for the diagnosis of HF in patients with shortness of breath and to strongly predict patient deaths (Baggish et al. 2006). Furthermore, a multicenter, international study showed that NT-proBNP testing was valuable for diagnostic evaluation and short-term prognosis estimation in patients with shortness of breath due to suspected or confirmed acute HF and should establish broader standards for use of the NT-proBNP in such patients (Januzzi et al. 2006). A major concern about the widespread use of the marker had been previous assertions that kidney disease, which is very common in patients with HF, might confound the results of NT-proBNP testing, since levels of the biomarker were higher among those with reduced renal function. However, NT-proBNP blood remains effective in patients with chronic kidney disease. Besides the diagnostic value of NT-proBNP, it was an even stronger predictor of death in breathless patients with significant renal insufficiency, emphasizing the fact that the biomarker is likely detecting a true signal of cardiac disease in these patients. This is a big step forward in the understanding of the optimal application of NT-proBNP measurement, as it removes one of the biggest obstacles that remained for the biomarker.

Use of BNP levels for rapid and accurate diagnosis of HF, a major unmet clinical need in primary care, was evaluated in a randomized controlled trial in Switzerland (Burri et al. 2012). The primary endpoint was total medical cost at 3 months and secondary endpoints were diagnostic certainty, time to appropriate therapy, functional capacity, hospitalization and mortality. Results showed that the use of BNP levels in primary care did not reduce total medical cost, but improved some of the secondary endpoints including diagnostic certainty and time to initiation of appropriate treatment. A study has examined the ease of use of Alere Heart Check BNP measurement system for point-of-care and found that BNP concentration measurements obtained by patients show excellent correlation with those obtained by healthcare providers (Lang et al. 2014).

Oxidative Stress as Biomarker of Heart Failure

Nanosensing techniques have been developed to monitor the physiology of NO in the beating heart in vivo. These methods involve the application of nanosensors to monitor real-time dynamics of NO production in the heart as well as the dynamics of

oxidative species (oxidative stress) produced in the failing heart. Use of nanotechnology has demonstrated that African Americans have an inherent imbalance of NO, O2-, and ONOO- production in the endothelium. The overproduction of O2- and ONOO- triggers the release of aggressive radicals and damages cardiac muscle (necrosis), which may explain why African Americans are at greater risk for developing cardiovascular diseases, such as HPN and HF, and are more likely to have complications than European Americans. Potential therapeutic strategies to prevent or ameliorate damage to the heart during cardiac events are prevention of O2- and ONOO- production, NO donors, and scavenging of O2- (antioxidants).

Future Prospects for Biomarkers of Heart Failure

A biomarker profile may be a valuable addition to the conventional classification of heart failure according to underlying pathological conditions. Rather than focus on an individual biomarker, an approach involving multiple biomarkers is useful for refining risk stratification among patients with acute coronary syndromes. For example, the use of data on BNP together with troponin has been shown to achieve better risk stratification than that obtained with either biomarker alone. The accuracy of risk prediction is enhanced when a natriuretic peptide is coupled with other biomarkers of myocardial stress or inflammatory biomarkers. The next step might be to obtain a profile by measuring representatives of distinct classes of biomarkers. The use of biomarkers for targeted therapy and for monitoring therapy requires extra effort, which would be worthwhile as it would likely enhance their clinical value. Currently, only the natriuretic peptides appear to be useful for these purposes. New approaches in bioinformatics, including the use of artificial neural networks, will probably be needed to assist in data analysis and its clinical application.

Biomarkers for Atherosclerosis

Atherosclerotic cardiovascular disease is the primary cause of morbidity and mortality, but due to lack of sensitive and specific early biomarkers, the first clinical presentation of more than half of these patients is either myocardial infarction or death. Despite appropriate evidence-based treatments, recurrence and mortality rates remain high even among patients with long established atherosclerotic cardiovascular disease.

9p21–3 Locus and Coronary Atherosclerosis

Various studies suggest that the 9p21–3 locus may influence susceptibility to myocardial infarction. A systematic review and meta-analysis was performed to assess whether this locus is associated with severity of coronary atherosclerosis and adverse clinical outcomes in those with known coronary disease (Munir et al. 2014).

Although subjecta with coronary atherosclerosis who carry the high risk genotype of the 9p21–3 allele may be more likely to have multi-vessel CAD, the effect of this allele on CAD progression and disease specific clinical outcomes were not observed in the study possibly due to diminishing genetic risk following dietary modification and therapy.

Adipocyte Enhancer-binding Protein 1

Peroxisome proliferator-activated receptor γ1 (PPARγ1) and liver X receptor α (LXRα) play pivotal roles in macrophage cholesterol homeostasis and inflammation, key biological processes in atherogenesis. Adipocyte enhancer-binding protein 1 (AEBP1) is a transcriptional repressor that impedes macrophage cholesterol efflux, promoting foam cell formation, via PPARγ1 and LXRα down-regulation. Contrary to AEBP1 deficiency, AEBP1 overexpression in macrophages is accompanied by decreased expression of PPARγ1, LXRα, and their target genes ATP-binding cassette A1, ATP-binding cassette G1, apolipoprotein E, and CD36, with concomitant elevation in IL-6, TNFα, monocyte chemoattractant protein 1, and inducible NO synthase levels. AEBP1, represses PPARγ1 and LXRα in vitro. Expectedly, AEBP1-overexpressing transgenic (AEBP1TG) macrophages accumulate considerable amounts of lipids compared with AEBP1 nontransgenic macrophages, making them precursors for foam cells. These in vitro and ex vivo experimental data strongly suggest that AEBP1 plays critical regulatory roles in macrophage cholesterol homeostasis, foam cell formation, and proinflammation. AEBP1 may be critically implicated in the development of atherosclerosis. As a biomarker for atherosclerosis, AEBP1 may serve as a molecular target toward developing antiinflammatory, antiatherogenic therapeutic approaches.

Gene Signatures on Leucocytes as Biomarkers of Atherosclerosis

Diagnosis of subclinical atherosclerosis is often difficult since those affected are asymptomatic. Imaging methods for plaque detection in human coronary arteries are expensive and associated with the risk of adverse events. To overcome these limitations, a molecular prediction technique has been developed for detecting atherogenic risks in patients using multi-gene expression biomarkers on leukocytes (Cheng et al. 2012). The investigators discovered 356 expression biomarkers reported in publications of microarray data, which showed significant differential expression between genome-wide microarray data of monocytes from patients with familial hyperlipidemia and increased risk of atherosclerosis compared to normal controls. These biomarkers were further triaged with 56 biomarkers known to be directly related to atherogenic risks. A COXEN algorithm was also applied to identify concordantly expressed biomarkers between monocytes and each of three different cell types of leukocytes. A multi-gene predictor was then developed using all or three subsets of these 56 biomarkers on the monocyte patient data. These predictors were then applied to multiple independent patient sets from three cell types of

leukocytes (macrophages, circulating T cells, or whole white blood cells) to predict patients with atherogenic risks. When the 56 predictors were applied to the three patient sets from different cell types of leukocytes, all significantly differentiated patients with atherogenic risks from healthy people in these independent cohorts. Concordantly expressed biomarkers identified by the COXEN algorithm provided slightly better prediction results. These finding show the potential of molecular prediction of atherogenic risks across different cell types of leukocytes. If current findings are validated, atherosclerosis biomarkers can be used in a clinical diagnostics setting. The type of platform used to test these biomarkers may depend on the minimum number of informative genes that are needed to distinguish between the high- and low-risk individuals. A specific number of genes can be combined into a simpler assay such as RT-PCR for use as a diagnostic tool.

Ghrelin as a Biomarker of Atherosclerosis

Ghrelin, a peptide hormone from stomach, stimulates food intake and decreases fat utilization. Ghrelin binds to growth hormone secretagogue receptor (GHSR). GHSR density is upregulated in atherosclerotic lesions. Ghrelin concentrations and carotid artery atherosclerosis are positively associated in males even after adjustment for the commonly recognized risk factors of atherosclerosis. Experimental and prospective studies are warranted to elucidate the role of ghrelin in atherosclerosis.

Imaging Biomarkers of Hypercholesterolemia/Atherosclerosis

Coronary artery calcium (CAC) and carotid intima-media thickness (IMT) are noninvasive measures of atherosclerosis that consensus panels have recommended as possible additions to risk factor assessment for predicting the probability of cardiovascular disease (CVD) occurrence. Atherosclerosis progression from childhood into old age has been followed by measuring intima-media thickness in subjects with familial hypercholesterolemia (FH) and measurement of arterial wall thickness is used as a surrogate marker for atherosclerosis.

A study has investigated the determinants of coronary and carotid subclinical atherosclerosis, aortic stiffness and their relation with inflammatory biomarkers in FH (Martinez et al. 2008). The clinical parameters poorly explained IMT, CAC and carotid-femoral pulse wave velocity variability in FH. Furthermore, imaging biomarkers and inflammatory biomarkers presented a poor agreement in degree of their severity for CHD prediction.

Inflammatory Biomarkers of Atherosclerosis

Scientific evidence and findings from both clinical and population studies indicate that chronic inflammation contributes to the development and progression of cardiovascular diseases. Inflammation has been implicated in all stages of atherosclerosis

and elevated serum inflammatory biomarkers have been used to assess cardiovascular risk and response to therapy. However, most of the current known inflammatory biomarkers are not useful in screening for atherosclerotic disease. C-reactive protein, sedimentation rate, and fibrinogen are not derived from the vasculature and may signal inflammation in any organ. It is also possible that due to heterogeneity among the population at risk, a single marker cannot provide sufficient information for accurate prediction of disease. Therefore, there is a need for identification of inflammatory markers that are more specific to vascular disease and can be used for highly sensitive and specific assays capable of detecting and quantifying atherosclerotic cardiovascular disease. Measurement of multiple circulating disease-related inflammatory factors may be more informative, enabling the early identification of vascular wall disease activity.

Lipid-modified Proteins as Biomarkers of Atherosclerosis

The biological function lipid-modified proteins depends on the identity of the attached lipid. At least five different lipid modifications of cysteines, glycines and other residues on the COOH- and NH(2)-terminal domains have been described. Available evidence suggests that lipid-modified proteins are directly involved in different steps of the development of lesions of atherosclerosis, from leukocyte recruitment to plaque rupture, and their expression or lipid modification are likely altered during atherogenesis. Several lipid-modified proteins can be used as biomarkers for atherosclerosis.

Lp-PLA2 as Biomarker of Atherosclerotic Heart Disease

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a member of the phospholipase A2 superfamily, a family of enzymes that hydrolyze phospholipids. Circulating Lp-PLA2 is a marker of inflammation that plays a critical role in atherogenesis; its inhibition may have antiatherogenic effects. Epidemiological data have consistently demonstrated the association of increased levels of Lp-PLA2 with increased risk of coronary heart disease. Polymorphisms of the Lp-PLA2 gene have been reported, with varying significance, in Japanese and Caucasian populations. Overall, epidemiological studies suggest that measurement of Lp-PLA2 in plasma may be a useful biomarker for identifying individuals at high risk for cardiac events. PLAC test (Diazyme) for detection of Lp-PLA2 in plasma is effective as a predictive biomarker of risk for cardiovascular disease and approved by the FDA.

Metabolomic Profile in Hypercholesterolemia

Metabolic imbalance has been characterized in a strain of heritable hyperlipidemic rabbits as a model of hypercholesterolemia. Using a MS-based system, scientists at Human Metabolome Technologies measured a total of 335 metabolites in plasma

and tissues from hyperlipidemic and healthy control rabbits (Ooga et al. 2011). From the comparison between two metabolomic profiles, pathophysiological features including glutathione and phosphatidylcholine metabolism indicated the occurrence of oxidative stress in several tissues. Imbalance of purine catabolism in the liver shed light on the transcriptional activation of xanthine oxidase, which can absorb or possibly trigger oxidative stress. This system was also applied to assess the therapeutic effects of simvastatin, which restored the metabolism to the healthy state. These changes were considered to be due to the pleiotropic action of statin, including antioxidant effects, rather than its main inhibitory action on cholesterol biosynthesis.

Nitric Oxide Impairment and Atherosclerosis

The role of nitric oxide (NO) in the cardiovascular system and in atherosclerosis has been described in detail in a special report (Jain 2017). Many studies have shown that NO is a major antioxidant that serves to block oxygen-free radicals, which, among other things, create oxidized low-density lipoprotein (LDL) cholesterol that damages the endothelium and leads to atherosclerosis. The measurement of NO bioavailability is of great clinical interest in the assessment of vascular health. However, NO is rapidly oxidized to form nitrite and nitrate and thus its direct detection in biological systems is difficult.

Oxygen Free Radicals as Biomarkers of Atherosclerosis

Excessive production and/or inadequate removal of ROS, especially superoxide anion, have been implicated in the pathogenesis of many cardiovascular diseases, including atherosclerosis, hypertension, diabetes, and in endothelial dysfunction by decreasing NO bioactivity. Vascular aging may be related to oxidative stress. Age-related morphologic changes in large resistance vessels include an intima-media thickening, increased deposition of matrix substances, thus ultimately leading to a reduced compliance. Vascular aging is mainly characterized by an impaired endothelium-dependent vasorelaxation. The expression of endothelial nitric oxide synthase (eNOS), producing vasodilatatory NO, is markedly upregulated with increasing age. However, vasorelaxation is impaired, as the production of ROS such as superoxide (O2-), concomitantly increases.

Proteomic Profiles of Serum Inflammatory Biomarkers of Atherosclerosis

Protein microarray-based measurements of abundant circulating proteins have been carried out to identify biomarkers of atherosclerotic disease. Signature pattern derived from simultaneous measurement of these biomarkers, which represent diverse atherosclerosis-related biological processes, will likely add to the specificity needed for diagnosis of atherosclerotic disease. Data suggest that quantification of

multiple disease-related inflammatory proteins will provide a more sensitive and specific method for assessing atherosclerotic disease activity in humans, and identify candidate biomarkers for such studies. Because the vascular tissue is not readily accessible, identification of protein markers in the serum can have practical implications in developing diagnostic tools for diagnosis of coronary artery disease in humans. A detailed microarray based picture of the transcriptional landscape in the diseased tissue, would be useful for assessing upstream components in the pathways that lead to inflammatory mediator expression, which is the first step in developing highly targeted therapeutics. Serum biomarker assays can then be used to assess the effects of such therapeutics.

Biomarkers of Coronary Heart Disease

Apolipoproteins as Risk Factors for Coronary Heart Disease

Apolipoproteins AI and B are structural components of lipoprotein particles, and also determinants of the metabolic fate of the encapsulated lipid, cholesterol and triglyceride. Development of accurate assays for these apolipoproteins has opened the way for their use as predictors of coronary heart disease (CHD) risk. Interpretation of AI and apo B levels is best undertaken with background knowledge of the metabolic status of an individual, especially the lipolytic capacity as reflected in the triglyceride concentration. Those with raised triglyceride, in general, not only have an elevated apo B/apo AI ratio, but also apo B-containing lipoproteins with a prolonged residence time, providing more opportunities for modification. Assessment of apolipoprotein levels is an aid to risk prediction and can be useful in tailoring treatment. An inverse relationship between the concentration of high-density lipoprotein (HDL) cholesterol and the risk of developing cardiovascular is well established. There are several documented functions of HDLs that may contribute to a protective role of these lipoproteins. These include the ability of HDLs to promote the efflux of cholesterol from macrophages and foam cells in the artery wall and to antiinflammatory/antioxidant properties of these lipoproteins. The fact that the main apolipoprotein of HDLs, apoA-I, plays a prominent role in each of these functions adds support to the view that apoA-I should be measured as a component of the assessment of cardiovascular risk in humans.

Results from some epidemiological studies and statin trials suggest that apolipoprotein B-100 (apoB), with or without apoA-I, is superior to LDL cholesterol in predicting coronary events. Measurements of apolipoproteins are internationally standardized, automated, cost-effective and more convenient and precise than those for LDL cholesterol. ApoB may also be preferable to the measurement of non-HDL cholesterol. Measurement of apolipoproteins (apoB and possibly apoA-I) should be added to the routine lipid profile (cholesterol, triglycerides and high-density lipoprotein cholesterol) for assessing the atherogenic potential of lipid disorders, particularly dyslipidemias characterized by an elevation in plasma triglycerides.

Apolipoproteins, especially apoB, could also replace the standard 'lipid profile' as a target for therapy in at-risk patients.

According to guidelines of the American College of Cardiology and American Heart Association, attenuated associations of lipoprotein particle measures with CHD after the adjustment for lipids indicate that their measurement does not detect risk that is unaccounted for by the standard lipid panel (Steffen et al. 2015). However, the possibility that lipoprotein measures may identify CHD risk in a subpopulation of individuals with normal cholesterol, but elevated lipoprotein particle numbers cannot be ruled out.

CRP as Biomarker of Risk for Coronary Heart Disease

CRP levels have been considered as a biomarker of CAD but this was somewhat controversial until recently. In an epidemiologic study, among those whose CRP levels were among the top one-fourth of participants, there was a 66% increased adjusted risk of developing coronary artery disease compared to the risk experienced by those whose levels were in the lowest fourth (Boekholdt et al. 2006). When fatal coronary artery disease risk was separately examined, it was found to be nearly three times greater among participants whose levels were in the top fourth of CRP levels than those whose levels were lowest, making CRP a more predictive risk factor than even smoking and diabetes among this group. The results indicate that the predictive value of CRP plasma levels may be stronger for mortality than for total coronary artery disease incidence.

High sensitivity CRP (hsCRP) is an alpha globulin that is synthesized in the liver and present as a trace constituent in the blood. Levels of hsCRP rise during general, nonspecific response to a wide variety of diseases, therefore elevated hsCRP is not specific to any particular disease. Despite this limitation, hsCRP is a useful indicator of the inflammatory processes that usually accompany cardiovascular disease and, when monitored in conjunction with the other cardiac biomarkers, can aid in diagnosis. Prospective studies have shown hs-CRP to be a predictor of increased cardiovascular risk in both men and women and that it may be a better predictor of the risk for heart attacks than cholesterol. Evidence suggests patients with elevated hs-CRP and normal cholesterol levels are at greater risk than those with normal hs-CRP and high cholesterol levels. Reduction of levels of hsCRP at 30 days and 4 months after acute coronary syndrome are independently associated with long-term survival (Morrow et al. 2006). Patients treated with more aggressive statin therapy are more likely to achieve lower levels of hsCRP.

High Level of Blood Ceramides as a Biomarker of CHD

High blood concentrations of ceramides, metabolites of sphingomyelin (lipid species enriched in the outer leaflet of the plasma membrane and cell organelles of all tissues), are a biomarker of development and progression of CHD. Results of a

clinical study showed that individuals with the highest levels of blood ceramides had a 3- to 4-times greater risk of having a cardiovascular event compared with those with the lowest ceramide score, regardless of their LDL cholesterol level or obstruction in the coronary arteries (Meeusen et al. 2017). Evaluation of ceramide levels in patients who are not at immediate risk for CHD events may help cardiologists decide who could benefit from proactive and preventive treatment, such as statins, or lifestyle changes to prevent serious CHD. A test for ceramides is commercially available from Mayo Medical Laboratories.

Of the ceramide species, Cer(d18:1/18:0) has the strongest association with incident major adverse cardiovascular events among apparently healthy individuals (Havulinna et al. 2016). These results should encourage more detailed analyses of ceramides in cardiovascular pathobiology. It is hypothesized that higher plasma concentrations of the long chain ceramide species C22:0 and C24:0 will be associated with decline in verbal memory performance and overall cognitive performance as assessed by a standardized battery of cognitive tests recommended for the investigation of vascular cognitive impairment (). This hypothesis will be tested in a clinical trial (NCT01625754).

Impairment of EPCs by Oxidative Stress as a Biomarker of Disease

Circulating endothelial progenitor cells (EPCs) in adult human peripheral blood have been extensively studied as biomarkers for assessing the risk of cardiovascular disease in human subjects and as a potential cell therapeutics for vascular regeneration. EPCs are exposed to oxidative stress during vascular injury as residents of blood vessel walls (e.g. coronary arteries) or as circulating cells homing to sites of neovascularization. Given the links between oxidative injury, endothelial cell dysfunction, and vascular disease, recent investigation has focused on the responses of EPCs to oxidant stress and the molecular mechanisms that control redox regulation in these specialized cells. Various cell and flow cytometric techniques have been used to define and isolate EPCs from circulating blood and the current human and mouse genetic data, which offer insights into redox control in EPC biology and angiogenesis (Case et al. 2008). EPC responses to oxidant stress may be a critical determinant in maintaining the integrity and function of the cardiovascular system and perturbations of redox control in EPCs may lead to human diseases. High cholesterol causes increased oxidative stress, impairing the function of EPCs. In addition to being implicated in cardiovascular diseases, oxidative stress is also a factor in diabetes. A comprehensive understanding of how oxidative stress, the biochemical modification of cells, impairs EPC function may lead to antioxidant therapy to prevent cardiovascular disease. These strategies will need to be applied early in the disease when preventing oxidative damage is a possibility because once the damage has occurred it may not be reversible. Eventually it should be possible to do a simple blood test to measure EPCs to determine the risk for cardiovascular disease.

Role of TNF in Acute Coronary Syndromes

Tumor necrosis factor (TNF) and related molecules are involved in the development of acute coronary syndromes (ACS), particularly CD40 ligand-CD40 interaction, but several other members of the TNF family seem also promote immune-mediated coronary atherosclerotic plaque instability, including LIGHT (receptor activator of nuclear factor κB ligand) and TNF-α (Aukrust et al. 2011). TNFs are potent regulators of inflammation and cell survival and consist of 20 ligands that signal through 29 different receptors. TNF-related pathways could contribute to the non-resolving inflammation that characterizes atherosclerosis, representing pathogenic loops that are operating during plaque rupture and the development of ACS. A study investigated the release of inflammatory cytokines including TNF-α during the very early phase (first 24 h) of ACS (Brunetti et al. 2011). TNF-α levels were associated with a worse prognosis at follow-up. These TNF-related pathways could also represent new targets for therapy in this disorder.

Serum Parathyroid Hormone as Biomarker of CHD

A study has investigated the relationship between serum parathyroid hormone (PTH) level and common risk factors of CHD such as age, gender, cholesterol, glycosylated hemoglobin (HbA1c), hypertension, history of diabetes, smoking, and body mass index (Zhao et al. 2014). Serum PTH levels were positively correlated with high diastolic BP and HbA1c, and although not related to other risk factors of CHD it is also an independent risk factor for CHD. These results provide evidence that serum PTH level may be involved in the pathogenesis of CHD. Thus, PTH could be used as an important biomarker in the diagnosis of CHD as well as for identification of cases of hypertension that would benefit from lowering of BP as it links hypertension with CHD.

Serum Stem Cell Factor as a Biomarker of CHD

Stem cell factor (SCF) is one of the growth factors that are important in recruiting vascular progenitor cells, which play an important role in vascular repair in cardiovascular disease. In a study on patients who had a coronary event, lower SCF levels were associated with more severe coronary disease, less fibrous atherosclerotic plaques and an increased incidence of heart failure (Wigren et al. 2016). Expression of the SCF receptor c-kit was demonstrated in the subendothelial layer and fibrous cap of human atherosclerotic plaques. These findings suggest that the SCF-c-kit pathway may be a promising biomarker and therapeutic target in cardiovascular disease.

VILCAD Biomarker Score for Prediction of Long-term Mortality in CHD

There are only a few algorithms for predicting the long term risk of CHD. The VIenna and Ludwigshafen CAD (VILCAD) risk score was one of the first scores specifically tailored for this clinically important patient population. A study has refined risk prediction in stable CHD and created a new version of this prediction model that encompasses various pathophysiological pathways (Kleber et al. 2014). Patients with stable CHD from the LUdwigshafen RIsk and Cardiovascular health (LURIC) study were followed up for a median 9.8 years to find if the predictive power of the VILCAD score could be improved by the addition of novel biomarkers. Additional biomarkers were selected in a bootstrapping procedure based on Cox regression to determine the most informative predictors of mortality. The final multivariable model included nine clinical and biochemical markers: age, sex, left ventricular ejection fraction (LVEF), heart rate, N-terminal pro-BNP, cystatin C, renin, ^{25}OH-vitamin D3 and hemoglobin A1c. The extended VILCAD biomarker score achieved a significantly improved C-statistic and net reclassification index compared to the original VILCAD score. Omitting LVEF, which might not be readily measureable in clinical practice, slightly reduced the accuracy of the new BIO-VILCAD score but still significantly improved risk classification. VILCAD biomarker score based on routine parameters complemented by novel biomarkers outperforms previous risk algorithms and enables more accurate classification of patients with stable CHD, enabling physicians to choose more personalized treatment regimens for their patients.

Biomarkers for Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is defined as a group of diseases characterized by a progressive increase in pulmonary vascular resistance leading to right ventricular failure and premature death. The cause of primary pulmonary hypertension is not definitely known, but HSV-8 infection in immunocompromised individuals has been linked to it. There are several causes of secondary pulmonary hypertension, but it is usually due to an underlying heart condition, lung disease or pulmonary embolism. The diagnostic procedures include ECG, chest X-ray, Doppler echocardiography, pulmonary function tests, arterial blood gas analysis, computed tomography of the lungs, and pulmonary angiography. Biomarkers have been proposed to reliably monitor the clinical course.

Plasma BNP is elevated in cardiac right ventricular (RV) dysfunction and plays a key role in protecting the body from volume overload by maintaining renal function and sodium balance. Studies in patients with PAH have demonstrated that plasma BNP levels are raised proportionally to the extent of RV dysfunction. There is growing evidence that BNP may be a potential biomarker for PAH in screening for occult disease, diagnostic evaluation, prognosis, and estimating a response to therapy. Additionally, augmentation of the natriuretic peptide system through

exogenous administration of BNP or by preventing its degradation may be a promising option for the management of decompensated RV failure. Because plasma BNP levels rise in a variety of cardiopulmonary conditions and are affected by several physiological factors, BNP interpretation must not occur in isolation but rather within the context of clinical diagnosis.

University of Colorado scientists have devised effective and precise algorithms for sub-typing PAH pathologies and prognosis by use of a noninvasive blood sample with a panel of indicative biomarkers. Microarray expression was performed, and the expression profiles were analyzed for consistent and predictive differences in gene expression. They identified a signature set of 106 genes that discriminated with high certainty between patients with PAH and normal individuals. The results of the microarray analysis were retrospectively and prospectively confirmed by quantitative PCR for 2 of the 106 genes. Supervised clustering analysis generated a list of differentially expressed genes between patients with idiopathic and secondary causes of PAH. These findings may have important implications toward diagnosis, screening, and pathogenesis of this disease. Ultimately, the diagnostic platform would ideally be based upon a high throughput, low cost system detecting the known and validated changes in gene expression of biomarkers associated with the disease of interest. This format would be precise, accurate, scalable, portable, and widely feasible. Alternatively, the technology could be rolled into a more extensive panel of similar diagnostics, i.e. a broad pulmonary biomarker array on a probe array or Affymetrix chip, for more extensive applications in clinical diagnosis, drug development, or biomedical research. The technology has potential applications in clinical drug development, diagnostics (from risk-factor determination to disease staging), and personalized medicine. This technology is available for licensing.

Biomarkers of Abdominal Aortic Aneurysm

Abdominal aortic aneurysm (AAA) is the term used for significant ballooning of the aorta, the largest artery in the body, which may lead to rupture. AAA is the cause of more than 15,000 deaths each year in the US placing it as the 15th leading cause of death. Patients considered high-risk for AAA include older men with a history of cigarette-smoking, high blood pressure and cholesterol and with a family history of the disease. AAAs are difficult to diagnose because of their location deep within the abdomen, and are usually only detected by chance, when ultrasound, CT or MRI imaging techniques are performed for other reasons. Because these imaging techniques are relatively expensive, they are not regularly performed to search for aneurysms, even in high-risk patients. AAAs may also be detected when they become very large and rupture or are in immediate danger of rupturing, in which case emergency surgery is required and is often unsuccessful in saving the patient. Elective surgical treatment is recommended on the basis of an individual's risk of rupture, which is predicted by AAA diameter. However, the natural history of AAA differs between patients and a reliable and individual predictor of AAA

Table 15.3 Biomarkers of abdominal aortic aneurysm

Biomarkers originating from aortic extracellular matrix
Aminoterminal propeptide of type III procollagen
Cystatin C (decreased)
Elastase-α1-antitrypsin complexes
Elastin peptides
Matrix metalloproteinases
TGF-β
Biomarkers originating from vascular smooth muscle cells
Calponin
Creatine kinase
Smooth muscle myosin heavy chain
Biomarkers of coagulation
d-dimer
Tissue plasminogen activator
Biomarkers of inflammation
C-reactive protein

progression (growth and expansion rates) has not been established. Several biomarkers whose circulating levels increase, with some that decrease, in patients affected by AAA are listed in Table. However, they have yet to meet the triad of biomarker criteria: biological plausibility, correlation with AAA progression, and prediction of treatment effect on disease outcome. Table 15.3 lists biomarkers of abdominal aortic aneurysm.

Results of a study using transcript biomarker panel for the identification of ascending thoracic aortic aneurysms by whole blood qRT-PCR analysis suggest that to properly identify an aneurysm of ≥4 cm, at least 3 of 5 biomarkers must be elevated >1.5-fold (Black et al. 2013): four and a half LIM domains protein 2 (FHL2), collagen α1 (IX), and collagen α2 (V), whereas collagen α1(I), collagen α1(III) were less important. If a cost-effective screening tool could detect aneurysms early, it would significantly reduce aneurysm-related mortality. Biomarkers of aortic dissection, could be used to screen patients with symptoms, to identify patients at higher risk of aortic dissection and for prognostic stratification of affected patients (Morello et al. 2014).

Seeing a need for a more widely available screening tool, scientists at University of Virginia have conducted research to develop an easy, clinical diagnostic test to screen patients for abdominal aortic aneurysms before their conditions become dire. They identified 119 protein-based biomarkers in the plasma that could help alert physicians to the presence of an an AAA with a simple blood test. For further development and commercialization of their technology, they have licensed it to Ortho Clinical Diagnostics that would allow testing of the aneurysm biomarkers on a much greater scale.

Biomarkers of Thrombotic Disorders

Biomarkers of Arterial Thromboembolism

Thrombin is a serine protease and regulator of hemostasis that plays a critical role in the formation of thrombosis, or obstructive blood clots, which is a life-threatening condition associated with numerous diseases such as atherosclerosis and stroke. Thrombi in heart disease may embolize to the brain or peripheral arterial system. Blood clots occur from the formation of fibrin, controlled by the enzyme thrombin, in a complex cascade of protein interactions. Some patients are at greater risk for clotting, but existing blood tests are not consistently able to detect the formation of new clots. Current tests for blood clotting are indirect and although they are capable of detecting the breaking down of a clot, they cannot detect its initial formation. Efforts are being made to discover biomarkers of throbus formation.

Nanoparticles as Synthetic Biomarkers of Thrombus Formation

In an experimental study, thrombin-sensitive peptide substrates were designed to conjugate to the surface of FDA-approved iron oxide nanoparticles for detecting thrombi in living animals (Lin et al. 2013). Following intravenous infusion, these "synthetic biomarkers" survey the host vasculature for coagulation and when they encounter thrombin, the peptides are cleaved at a specific location, releasing fragments (tags) that are then excreted in urine. The urine samples are then treated with antibodies specific to the peptide fragments. Tested on mice, the researchers found that the amount of tags found in the urine was directly proportional to the level of blood clotting in mouse lungs. Using a thromboplastin-induced mouse model of pulmonary embolism, the authors showed that urinary biomarker levels differentiate between healthy and thrombotic states and correlate closely with the aggregate burden of clots formed in the lungs. These results demonstrate that synthetic biomarkers can be engineered to sense vascular diseases remotely from the urine and may enable POC detection of thrombi. A paper-based test for urine analysis, similar to a dipstick test for pregnancy, is in development.

Biomarkers of Venous Thromboembolism

Management of venous thromboembolism in the past was characterized by a high degree of complexity and lack of both efficacy and efficiency. The nonspecific clinical signs of acute pulmonary embolism (PE) and the limitations of earlier imaging procedures led to the development of numerous sophisticated, multistep diagnostic algorithms which, however, have proved extremely difficult to implement in clinical practice. The diagnosis of potentially life-threatening PE is still missed in many

patients who subsequently die of the disease without receiving appropriate treatment, while other patients unnecessarily undergo a invasive, time consuming procedures due to a vague, poorly documented clinical suspicion.

BNP and cTnT as Biomarkers of Outcome in Pulmonary Embolism

Combined use of BNP and cTnT may be useful in risk stratification of normotensive patients with acute PE. Patients with increased BNP and cTnT are at risk for adverse outcome. A prospective study in elderly patients showed that the overall complication rate during follow-up was 8.7% and hs-cTnT achieved the highest prognostic accuracy (Vuilleumier et al. 2015). At the predefined cut-off values, the negative predictive values of the biomarkers were >95%. For levels above the cut-off, the risk of complications increased 5-fold for hs-cTnT and 14-fold for NT-proBNP after adjustment for both clinical scores and renal function. Reclassification statistics indicated that adding hs-cTnT to the Geneva Prognostic Score or the Pulmonary Embolism Severity Index significantly improved the prognostic accuracy of both clinical scores. Thus, in elderly patients with non-massive PE, NT-proBNP or hs-cTnT was shown to be an adequate alternative to clinical scores for identifying low-risk individuals suitable for outpatient management.

D-dimer as Biomarker of Venous Thromboembolism

The widespread use of D-dimer testing in the outpatient setting, and particularly the technical advances of multidetector-row CT scan, had an enormous impact on our strategy for approaching patients with suspected PE. D-dimer testing was originally developed in the diagnosis of disseminated intravascular coagulation, but later turned out to be useful in venous thromboembolic (VTE) disorders. D-dimers are unique in that they are the breakdown products of a fibrin mesh that has been stabilized by factor XIII, which crosslinks the E-element to two D-elements. This is the final step in the generation of a thrombus. D-dimer ELISA assays rely on MAbs to bind to this specific protein fragment. D-dimer can also be used to monitor anticoagulation therapy for pulmonary embolism. Patients with an abnormal D-dimer level 1 month after the discontinuation of anticoagulation have a significant incidence of recurrent VTE, which is reduced by the resumption of anticoagulation. The optimal course of anticoagulation in patients with a normal D-dimer level has not been clearly established. Although D-dimer, a highly sensitive biomarker, is useful for excluding acute VTE, it lacks the specificity necessary for diagnostic confirmation.

Molecular Biomarkers of Venous Thromboembolism

As such, ongoing research efforts target and support the utility of plasma biomarkers as alternative to D-dimer for the diagnosis of VTE including selectins, microparticles, IL-10 and other inflammatory biomarkers. These molecular biomarkers may

also predict recurrence risk, guide length and modality of treatment, and predict which thrombi will resolve spontaneously or recanalize, thus potentially identifying patients who would benefit from more aggressive therapies than standard anticoagulation (Coleman and Wakefield 2012).

Genetic Biomarkers for Cardiovascular Disease

Biomarkers of Inherited Cardiomyopathies

Genes associated with cardiomyopathy were mentioned earlier in this chapter. Recent progress in genetic cardiomyopathy points to the potential value of genetic testing in helping the clinician to diagnose and understand the pathogenetic basis of the inherited cardiomyopathies. Genetic testing for cardiomyopathy has become a clinical reality in the form of sequencing of known disease-causing genes. Laboratory for Molecular Medicine of Partners Healthcare (Boston, MA) has developed Pan Cardiomyopathy Panel is comprised of 46 genes involved in inherited cardiomyopathies. Originally on a microarray platform, the panel now runs on the Illumina HiSeq platform, supplemented by some Sanger sequencing.

Hypertrophic cardiomyopathy is a common inherited heart muscle disorder associated with sudden cardiac death, arrhythmias and heart failure. Genetic mutations can be identified in ~60% of patients; these are commonest in genes that encode proteins of the cardiac sarcomere. Similar to other Mendelian diseases these mutations are characterized by incomplete penetrance and variable clinical expression. Our knowledge of this genetic diversity is rapidly evolving as high-throughput DNA sequencing technology is now used to characterize an individual patient's disease. In addition, the genomic basis of several multisystem diseases associated with a hypertrophic cardiomyopathy phenotype has been elucidated. Genetic biomarkers can be helpful in making an accurate diagnosis and in identifying relatives at risk of developing the condition (Coats and Elliott 2013).

Gene Mutations in Pulmonary Arterial Hypertension

Mutations in bone morphogenetic protein receptor type-2 (BMPR2) are found in about half of the patients with familial PAH. Because familial PAH is highly linked to chromosome 2q33, it is likely that the remaining 50% of family cases without exonic mutations have either intronic BMPR2 abnormalities or alterations in the promoter or regulatory genes. Also, only about 10% of patients with "sporadic" idiopathic PAH have identifiable BMPR2 mutations. Mutations in BMPR2 confer a 15–20% chance of developing PAH in a carrier's lifetime. Advances in genetic testing, presymptomatic screening, and biomarkers should permit early detection of disease in those at risk of PAH and allow trials of preventive therapy in carriers.

Gene Variant as a Risk Factor for Sudden Cardiac Death

Extremes of the electrocardiographic QT interval, a measure of cardiac repolarization, are associated with increased cardiovascular mortality. NOS1AP (CAPON) gene predisposes some people to abnormal heart rhythms, which leads to sudden cardiac death. Statistically significant findings were validated in two independent samples of 2646 subjects from Germany and 1805 subjects from the US Framingham Heart Study. NOS1AP, a regulator of neuronal nitric oxide synthase (nNOS), modulates cardiac repolarization. The gene, not previously discovered by traditional gene-hunting approaches, appears to influence significantly QT interval length as risk factor for sudden cardiac death. QT interval can be measured non-invasively with an EKG, and each person's QT interval, in the absence of a major cardiovascular event, is stable over time, making it a reliable measure. Approximately 60% of subjects of European ancestry carry at least one minor allele of the NOS1AP genetic variant, which explains up to 1.5% of QT interval variation.

Instead of focusing on so-called candidate genes with known functions that are highly suspect in heart beat rhythm, the researchers first focused on people who have extremely long or short QT intervals. They used subjects from two population-based studies, about 1800 American adults of European ancestry from the Framingham Heart Study of Framingham, Massachusetts, and about 6700 German adults from the KORA-gen study of Augsburg, Germany. They looked at SNPs that track with having a long or short QT interval. Only one particular SNP correlated with QT interval. That SNP was found near the NOS1AP gene, which has been studied for its function in nerve cells and was not previously suspected to play a role in heart function.

Identifying those at high risk for sudden cardiac death before fatalities occur has been challenging, both at the clinical and at the genetic level. In more than one third of all cases, sudden cardiac death is the first hint of heart disease. It is widely believed that many factors, genetic and environmental, contribute to irregular heartbeat and other conditions that may lead to sudden cardiac death. Now that variants of the NOS1AP gene have been correlated with QT interval length, the next project would be to figure out exactly how the DNA sequence variations alter the function of the gene, and how changes in gene function affects heart rhythm. Being able to identify predisposed individuals can save their lives by prescribing beta-blockers and other drugs that regulate heart rhythm, and even by implanting automatic defibrillators in those with the highest risk.

Genetic Biomarkers of Early Onset Myocardial Infarction

VAMP8, which is involved in platelet degranulation, and HNRPUL1, which encodes a ribonuclear protein are two SNP-biomarkers associated with an increased genetic risk for MI. Results of large scale studies with well-characterized samples from carefully selected patients allow the identification of genetic biomarkers for risk of early-onset MI, which could potentially be incorporated into individual risk

assessment protocol. The identification of these variants could improve understanding of disease mechanisms and suggest novel drug targets.

Genetic Biomarkers of Atherosclerosis

Finding genes that influence systemic levels of inflammatory biomarkers may provide insights into genetic determinants of atherosclerosis. Variance-component linkage analyses of blood levels of four biomarkers of vascular inflammation – CRP, IL-6, MCP-1, sICAM-1 – in extended families from the Framingham Heart Study showed that multiple genes on chromosome 1 may influence inflammatory biomarker levels and may have a potential role in development of atherosclerosis.

Dysfunction of endothelial lining is a primary cause of cardiovascular disease. Several genes that are involved in the development of endothelium of blood vessels have been identified. Lesions of atherosclerosis are isolated to atheroprotective and atheroprone regions. The blood flow is laminar in atheroprotective region and lesions do not form whereas the flow is turbulent in atheroprone regions leading to atherosclerosis. They studied the two regions by genome-wide transcriptional profiling using gene expression arrays from Life Technologies Corp and analyzed the differences in expression of transcription factors to identify potential biomarkers. They found that the transcription factor Kruppel-like factor 2 (KLF2) is selectively induced in endothelial cells exposed to a biomechanical stimulus characteristic of atheroprotected regions of the human carotid and this flow-mediated increase in expression occurs via a MEK5/ERK5/MEF2 signaling pathway (Parmar et al. 2006). Overexpression and silencing of KLF2 in the context of flow, combined with findings from genome-wide analyses of gene expression, demonstrate that the induction of KLF2 results in the orchestrated regulation of endothelial transcriptional programs controlling inflammation, thrombosis/hemostasis, vascular tone, and blood vessel development. These data also indicate that KLF2 expression globally modulates IL-1beta-mediated endothelial activation. KLF2 therefore serves as a mechano-activated transcription factor important in the integration of multiple endothelial functions associated with regions of the arterial vasculature that are relatively resistant to atherogenesis.

IL-1 Gene Polymorphism as Biomarker of Cardiovascular Disease

IL-1 is one of the body's most potent natural mechanisms for controlling the inflammatory response to injury. Inflammation is a crucial factor in the process that leads to heart disease and subsequent heart attacks. Certain variations in the IL-1 gene can amplify inflammation in the arteries, and make an individual 2–4 times more likely to develop clinically significant heart disease prior to age 60. These gene variations been implicated in essential hypertension and heart failure. The Gensona™ Heart Health Genetic Test (Interleukin Genetics) is the first and only IL1 gene test to identify an individual's predisposition for over-expression of inflammation and increased risk for cardiovascular disease.

IL-6R Signaling Pathway and Coronary Heart Disease

Persistent inflammation has been suggested to contribute to various stages in the pathogenesis of cardiovascular disease. Interleukin-6 receptor (IL-6R) signaling propagates downstream inflammation cascades. A functional genetic variant known to affect IL6R signaling has been studied to assess whether this pathway is causally relevant to coronary heart disease. Meta-analysis of collaborative studies using Illumina's Cardiochip showed that a SNP in the IL-6R gene was associated with lower risk of coronary heart disease and also altered inflammation-associated biomarkers in a way similar to the antiinflammatory drug tocilizumab designed to inhibit the inflammatory cytokine IL6R protein (IL6R Genetics Consortium Emerging Risk Factors Collaboration 2012). Tocilizumab is marketed as Actemra (Chugai) and RoActemra (Roche) for the treatment of rheumatoid arthritis. It was concluded that IL-6R signaling has a causal role in development of coronary heart disease and that IL-6R blockers could provide a therapeutic approach to prevention of the disease.

Kallikrein Gene Mutations in Cardiovascular Disease

All the components of the kallikrein-kinin system are located in the cardiac muscle, and its deficiency may lead to cardiac dysfunction. Muations of the kallikrein gene are associated with several diseases. In recent years, numerous observations obtained from clinical and experimental models of diabetes, hypertension, cardiac failure, ischemia, myocardial infarction and left ventricular hypertrophy have suggested that the reduced activity of the local kallikrein-kinin system may be instrumental for the induction of cardiovascular-related diseases. The cardioprotective property of the angiotensin converting enzyme inhibitors is primarily mediated via kinin-releasing pathway, which may cause regression of the left ventricular hypertrophy in hypertensive situations. The ability of kallikrein gene delivery to produce a wide spectrum of beneficial effects makes it an excellent candidate in treating cardiovascular diseases (Sharma 2006).

Kallikrein Gene and Essential Hypertension

Ten alleles with length and nucleotide sequence variations were identified in the regulatory region of human tissue kallikrein gene. There are polymorphisms in the regulatory region of human tissue kallikrein gene in the Chinese Han people. Differences in both allele frequencies and genotype frequencies between these two groups have provided evidence towards the association of hypertension with the polymorphisms in this studied site.

US Patent No. 5948 from Medical University of South Carolina (Charleston, SC) describes a biomarker for identifying a human subject as having an increased or decreased risk of developing essential hypertension. This is carried out by determining the presence in the subject of an allele in the promoter region of the tissue

kallikrein gene, which is correlated with an increased or decreased risk of developing essential hypertension. US Patent No. 6,747,140 describes the susceptibility to develop hypertension associated with a mutation in the kallikrein gene.

Mutations in the Low Density Lipoprotein Receptor Gene

Familial hypercholesterolemia is an autosomal dominant disease defined at the molecular level mainly by the presence of mutations in the low density lipoprotein (LDL) receptor gene. More than 600 mutations in the LDLR gene have been identified in patients with this disorder. One in 500 people is heterozygous for at least one such mutation, whereas only one in a million is homozygous at a single locus. Those who are heterozygous produce half the normal number of LDL receptors, leading to an increase in plasma LDL levels by a factor of 2 or 3, whereas LDL levels in those who are homozygous are 6–10 times normal levels. Affected individuals typically have cholesterol concentrations twice the population average for their age and sex. LDL is the major cholesterol-carrying lipoprotein in plasma and is the causal agent in many forms of coronary heart disease. Four monogenic diseases raise plasma levels of LDL by impairing the activity of hepatic LDL receptors, which normally clear LDL from the plasma.

There is an interest in developing diagnostic systems for LDL genotyping, which will enable identification of responders to statin therapy and those at increased risk of adverse drug reactions or patients.

Mutations within Several Genes that Code for Ion Channel

Inherited cardiac rhythm disorders are a group of genetically determined diseases due to mutations in genes coding for various cardiac ion channels. The most common cardiac ion channel disease is the long QT syndrome. Cardiac ion channel disorders may lead to sudden cardiac death. Prophylactic and life-saving therapies are available for many of these disorders. Therapy and risk stratification depend on the clinical presentation, the ECG pattern, and which gene is mutated. Genetic testing offers the opportunity to exclude individual family members as mutation carriers.

Following earlier studies of the involvement of some cardiac ion currents in adverse drug interactions, recent reports have identified not only the ion channel subunits involved but also a range of mutations and SNPs in ion channel genes that predispose to both drug-induced and familial cardiac arrhythmia.

Polymorphisms of the eNOS Gene and Angina Pectoris

Angina pectoris is chest pain due to transient myocardial ischemia. It may be a stable chronic manifestation with angina occurring on exertion or unstable form of angina with pain at rest, which may be due to coronary artery disease. Coronary artery spasm plays an important role in the pathogenesis of vasospastic angina, and

contributes to the development of several acute coronary syndromes. Endothelial nitric oxide synthase (eNOS) catalyzes the synthesis of nitric oxide (NO), which regulates vascular tone, and may be related to coronary vasospasm. Coronary spasm may be related to particular polymorphisms of the eNOS gene. The a/a or a/b genotype in intron 4 of eNOS (NOS4a) is a significant predictor of coronary spasm (Kaneda et al. 2006). In patients with NOS4a, both the induced and spontaneous contractions are augmented. Indicating that NOS4a could be a good marker for coronary artery spasm.

Lipoprotein (a) Genetics

Plasma lipoprotein(a) [Lp(a)] is a quantitative genetic trait with a very broad and skewed distribution which is largely controlled by genetic variants at the LPA locus on chromosome 6q27. Based on genetic evidence provided by studies conducted over the last two decades, Lp(a) is currently considered to be the strongest genetic risk factor for CHD. The CNV of kringle IV in the LPA gene has been strongly associated with both Lp(a) levels in plasma and risk of CHD, thereby fulfilling the main criterion for causality in a Mendelian randomization approach. Alleles with a low kringle IV copy number which together have a population frequency of 25–35% are associated with a doubling of the relative risk for outcomes, which is exceptional in the field of complex genetic phenotypes. The recently identified binding of oxidized phospholipids to Lp(a) is considered as one of the possible mechanisms that may explain the pathogenicity of Lp(a). Drugs that have been shown to lower Lp(a) have pleiotropic effects on other CHD risk factors and an improvement of cardiovascular endpoints is up to now lacking. However, it has been established in a proof of principle study that lowering of very high Lp(a) by apheresis in high-risk patients with already maximally reduced LDL cholesterol levels can dramatically reduce major coronary events (Kronenberg and Utermann 2013).

Polymorphisms in the Apolipoprotein C Gene

Apolipoprotein C3 (APOC3) is a component of remnant particles that is associated with high levels of triglycerides and thus remnant cholesterol. APOC3 increases plasma triglyceride levels by inhibiting hydrolysis of triglyceride-rich lipoproteins by lipoprotein lipase and by attenuating the uptake of triglyceride-rich remnant lipoproteins by the liver. Sequencing of the protein-coding regions of the human genome, the exome, has the potential to identify rare mutations of APOC3. Protein-coding regions of genes in participants of European or African ancestry in the Exome Sequencing Project were sequenced to determine whether rare mutations in coding sequence of APOC3 were associated with plasma triglyceride levels (Crosby et al. 2014). For mutations associated with triglyceride levels, the investigators evaluated their association with the risk of CHD. Three of the four mutations in the gene encoding APOC3 and associated with lower plasma triglyceride levels, were

loss-of-function mutations: a nonsense mutation (R19X) and two splice-site mutations (IVS2 + 1G → A and IVS3 + 1G → T). The fourth was a missense mutation (A43T). Approximately 1 in 150 persons in the study was a heterozygous carrier of at least one of these four mutations. Triglyceride levels in the carriers were 39% lower than levels in noncarriers, and circulating levels of APOC3 in carriers were 46% lower than levels in noncarriers. The risk of CHD among carriers of any rare APOC3 mutation was 40% lower than the risk among noncarriers.

Another sequencing study from Denmark found that three rare variants of APOC3 — R19X, IVS2 + 1G → A, and A43T — are associated with substantially reduced levels of nonfasting triglycerides and reduced risk of ischemic cardiovascular disease in the general population (Jørgensen et al. 2014). One limitation of this study is that although the risk of ischemic cardiovascular disease is consistently inversely related to plasma levels of HDL cholesterol in observational studies, clinical trials as well as genetic studies involving mendelian randomization have failed to establish a causal link between plasma levels of HDL cholesterol and the risk of ischemic cardiovascular disease. Nevertheless, the findings of this study are of potential clinical importance, because they suggest that APOC3 is a relevant drug target for reducing residual cardiovascular risk. Inhibition of APOC3 by antisense oligonucleotides was shown to reduce plasma levels of APOC3 and triglycerides in animal models and in a phase I human clinical trial (Graham et al. 2013).

Polymorphisms in the Apolipoprotein E Gene

Apolipoprotein E (APOE) acts as a ligand for the LDL receptor and has an important role in clearing cholesterol-rich lipoproteins from plasma. The ϵ2, ϵ3, and ϵ4 alleles of the apolipoprotein APOE gene encode three isoforms, apoE2, E3, and E4, respectively. The apoE isoforms circulate in different plasma concentrations, but plasma concentrations of the same isoform also differ between individuals. This genetic variation is associated with different plasma lipoprotein levels, different response to diet and lipid-lowering therapy, and a variable risk for cardiovascular disease. The E4 allele of the apolipoprotein E gene occurs in about 30% of the general population (E3 being the most common allele). Heterozygotes possessing alleles E3/E4 have cholesterol concentrations on average 10% higher than those of E3/E3 homozygotes because of the differential allelic effects on lipoprotein particle turnover. This difference extends to coronary events, although the risk associated with the E4 allele seems greater than is indicated from its effect on cholesterol levels. A third version of the gene, the E2 allele, occurs in its homozygous form. When combined with a second risk factor such as diabetes or hypothyroidism, the E2/E2 genotype results in type III dysbetalipoproteinemia, which is associated with a high risk of coronary and peripheral vascular disease and occurs in 0.01–0.02% of the population.

In old age, high plasma apoE levels precede an increase of circulating CRP and strongly associates with cardiovascular mortality, independent of APOE genotype and plasma lipids. ApoE polymorphism is also a risk factor for Alzheimer's disease.

Polymorphism in the Angiotensinogen Gene

The renin-angiotensin system plays a central role in health and disease but the determinants of renin-angiotensin system activity have not been fully elucidated. A common variant of the angiotensinogen gene (T235) predicts elevated levels of circulating angiotensinogen, and polymorphisms of this gene have been linked to physiologic responses and to the risk of cardiovascular disease There is an association between angiotensinogen M235 T polymorphism and coronary artery disease severity independently of other cardiovascular risk factors. T235 is also a genetic marker for early carotid atherosclerosis in a hypertensive population, which has been shown to regression under antihypertensive treatment.

Multiple Biomarkers for Prediction of Death from Cardiovascular Disease

Multiple biomarkers from different disease pathways may be involved in fatal outcome from cardiovascular disease. Uppsala Longitudinal Study of Adult Men (ULSAM), a community-based cohort of elderly men, has shown that a combination of biomarkers that reflect myocardial cell damage, left ventricular dysfunction, renal failure, and inflammation (troponin I, N-terminal pro–brain natriuretic peptide, cystatin C, and C-reactive protein, respectively) improved the risk stratification of a person beyond an assessment that was based on the established risk factors for cardiovascular disease (Zethelius et al. 2008).

In addition to biomarkers, various well-validated scoring systems based on clinical characteristics are available to help clinicians predict mortality risk, such as the Thrombolysis In Myocardial Infarction (TIMI) score and Global Registry of Acute Coronary Events score. A multimarker approach incorporating biomarkers and clinical scores will increase the prognostic accuracy.

Role of Biomarkers in the Management of Cardiovascular Disease

Biomarkers in the Diagnosis/Prognosis of Myocardial Infarction

According to the World Health Organization, the diagnosis of myocardial infarction (MI) requires that two out of the following three criteria be met:

1. Clinical history of ischemic type chest pain for at least 20 min.
2. Changes in serial ECG tracings.
3. Rise and fall of serum cardiac enzymes (biomarkers)

BNP/NT-proBNP and CRP are mentioned in the current guidelines of the European Society of Cardiology as appropriate biomarkers for risk stratification of MI, whereas clinical relevance of other novel biomarkers remains uncertain and necessitates further studies (Weber and Hamm 2008). According to a 2009 Henry Ford Hospital (Detroit, MI) study, many patients with MI have high levels of cardiac biomarkers in the blood for several months after leaving the hospital, with more shortness of breath and chest pain. The study examined a subset of patients in a 4500-patient heart attack registry from 24 US hospitals and found that 6 months later 9% percent had elevated levels of the TnT and 33% had elevated level of the BNP/NT. These elevated biomarkers were definitely associated with a reduced quality of life for patients and worse outcomes. This data raises two important issues; (1) whether the biomarkers are a sign of ongoing problems or a reflection of the past MI; and (2) whether closer monitoring of patients post-MI can help target the treatment to those who need it most.

An observational study has followed plasma levels of IL-1β, infarct size and left ventricular (LV) remodeling after acute ST segment elevation MI (STEMI) and a single occluded vessel successfully revascularized by primary percutaneous coronary intervention (Ørn et al. 2012). IL-1β levels after STEMI were strongly associated with impaired myocardial function and non-infarct LV mass after 1 y, suggesting a role for IL-1β as a predictor of maladaptive myocardial remodeling following reperfused MI.

Biomarkers for Prevention of Cardiovascular Disease

Preventive treatment for those most at risk of heart disease rather than those with the highest blood pressure or cholesterol values may be a more efficacious strategy for disease management. This depends on accurate biomarker-based risk assessment tools. The American College of Cardiology Foundation and the American Heart Association (ACCF/AHA) have released guidelines on the use of existing biomarkers (Greenland et al. 2010). A summary of the current ACCF/AHA recommendations is presented in Table 15.4. Despite the large number of studies examining a host of candidate biomarkers, only the assessment of family history of cardiovascular disease was granted a class I recommendation, the highest possible level.

An evidence-based model of heart disease risk was developed using the Framingham model with an additional five risk factors, including three of the newer blood biomarkers. This was applied to the adult population of the 3rd National Health and Nutrition Examination Survey cohort. Additionally, the selection criteria for therapeutic intervention from the Adult Treatment Panel III guidelines (for hyperlipidemia) and the 7th Report of the Joint National Committee (for hypertension) were applied to the same subjects. Of this cohort 54% qualified for at least one of these medications while 18% qualified for both. Using this 18% cutoff, the 18% of the subjects with the highest calculated heart disease risk were also identified using the developed risk model. Applying both drugs to the high-risk group

Table 15.4 Biomarkers for cardiovascular disease risk prediction

Biomarker	ACCF/AHA 2010 recommendation
Circulating	
Lipoprotein and apolipoprotein	III
Natriuretic peptide	III
C reactive protein	IIa: To help determine use of statins in men >50 years, women >60 years of age and LDL <130 mg/dL IIb: Intermediate-risk adults
Lipoprotein-associated phospholipase A2	IIb: Intermediate-risk adults
Haemoglobin A1C	IIb: In adults without known diabetes
Urinary albumin	IIa: In adults with diabetes or hypertension IIb: Intermediate-risk adults
Imaging	
Resting ECG	IIa: in adults with diabetes or hypertension IIb: in asymptomatic adults
Exercise ECG	IIb: Intermediate-risk adults
Echocardiogram	IIb: Asymptomatic adult with hypertension III: Asymptomatic adults
Stress echocardiogram	III
Flow-mediated dilatation	III
Ankle–brachial index	IIa: Intermediate-risk adults
Carotid intima–media thickness	IIa: Intermediate-risk adults
Coronary calcium score	IIa: Intermediate-risk adults IIb: Low- to intermediate-risk adults
Myocardial perfusion imaging	IIb: High-risk adults III: Low- or intermediate-risk adults
Other	
Family history	Ib

(one third the size of the guidelines group) achieved the same reduction in population risk (about one fourth) as applying the drugs to the guideline groups and required only half as many prescriptions. Intermediate results were found when an intervention group was identified by a combination of both high risk and high levels of risk factors. In this simulation, identifying patients by heart disease risk level resulted in substantially fewer people being treated with fewer drugs and achieving a similar reduction in disease risk.

The benefit of a biomarker-based risk model is that the newest risk factor information can be incorporated into the model and a more accurate assessment of risk made. The cost of the biomarkers is an added cost not encountered in the guidelines-based approach. However, this increase is more than compensated for by the 44% reduction in cardiovascular disease events in the high-risk group.

Lower levels of physical activity and higher levels of body mass index (BMI) are independently associated with adverse levels of nearly all lipid and inflammatory biomarkers of cardiovascular disease risk. High BMI shows stronger associations

with these biomarkers than physical inactivity. However, within BMI categories, physical activity is generally associated with more favorable cardiovascular biomarker levels than inactivity. Results of a study that compared the traditional prediction model for cardiovascular disease using total cholesterol with a version in which cholesterol was replaced with BMI suggest that BMI is a valuable alternative to cholesterol for risk prediction models (Faeh et al. 2012).

Research into circulating, genetic and imaging biomarkers to augment traditional methods of risk prediction of CVD has achieved only modest success so far. Emerging technologies in "omics" are now providing new platforms for biomarker discovery. Further research is required to identify new biomarkers to successfully stratify risk of CVD in low-risk populations, as well as to test whether management strategies based on biomarker testing are better than standard-of-care (Ge and Wang 2012).

C reactive Protein as Biomarker of Response to Statin Therapy

CRP levels are low in healthy young people – usually <1 mg/L of blood – but they rise with age, obesity, diabetes, smoking and a sedentary life. If people lose weight, stop smoking, exercise or take oral diabetes drugs, their CRP levels fall. But a third of the population has levels >3 mg/L, and levels that high have been associated with heart disease risk. Questions remain as to the role of CRP in the normal body. It was discovered about 70 years ago by scientists who were trying to understand why some streptococci caused disease and others did not. It is so named because it was found in the third band, called Band C, in a gel used to separate proteins. Then, about half a century ago, physicians noticed that CRP flooded the patient's blood after a heart attack, and for several years, the protein was used to help diagnose heart attacks.

Several studies have shown that reducing the levels of CRP, which plays a role in heart disease, may be as powerful a tool in slowing heart disease and preventing heart attacks and cardiac-related death as lowering cholesterol. Some of the participants are patients with severe heart disease who were taking high doses of statin drugs, which reduce both cholesterol and CRP. However, CRP independently predicts heart disease progression. Lower CRP levels are linked to a slower progression of atherosclerosis and fewer heart attacks and deaths and this effect is independent of the effect of lowering cholesterol. This is hard clinical evidence that reducing CRP is at least as important as lowering cholesterol. These findings indicate that physicians should monitor CRP levels in patients with severe heart disease and do whatever it takes, including giving high doses of the most powerful statins, to get levels below 2 mg/L blood.

However, more work is needed to prove that CRP directly causes heart disease. The studies so far involved only people with severe heart disease, it is unknown whether healthy people would benefit from reducing their CRP levels. It is also possible that CRP is a biomarker for some other abnormality that is corrected by statin

drugs to reduce heart disease risk. Even before these studies, evidence had been accumulating that CRP and heart disease are somehow linked. There were hypotheses to explain why the protein can cause plaque to develop in coronary arteries, lead plaque to burst open or bring on the formation of blood clots, which block arteries and cause heart attacks. Some pharmaceutical companies have started programs to develop drugs that make a specific target of CRP and prevent its synthesis. That CRP levels drop with exercise and weight loss has led some to argue that the protein is a biomarker of heart disease risk, not a cause. CRP is made in the liver and also in the walls of coronary arteries and possibly elsewhere in the body. Its levels, which can be measured with a simple blood test, often rise and remain high in patients who have chronic inflammation from conditions like rheumatoid arthritis, for example, or periodontal disease. Patients with chronic inflammation also have an increased risk of heart disease. The next step is to see if reducing CRP levels can prevent heart attacks in healthy people. A new study will enroll 15,000 people with normal cholesterol levels but higher-than-average levels of CRP, >2 mg/L blood. The participants will be randomly assigned to take either a statin or a placebo.

HSP72 and eNOS as Biomarkers of Cardioprotective Effect of HBO

In clinical studies hyperbaric oxygen (HBO) preconditioning consisting of 2.30-min intervals of 100% oxygen at 2.4 atmospheres absolute (ATA) prior to coronary artery bypass graft (CABG) surgery has been shown to improve left ventricular stroke work (LVSW) 24 h following CABG. Intraoperative right atrial biopsies were assessed using an ELISA for the expression of eNOS and HSP72 (Jeysen et al. 2011). In this study, no significant differences were observed between the groups with respect to the quantity of myocardial eNOS and HSP72. However, in the HBO Group, following ischemia and reperfusion, the quantities of myocardial eNOS and HSP72 were increased. This suggests that HBO preconditioning in this group of patients may be capable of inducing endogenous cardioprotection following ischemic reperfusion injury, which can be assessed by eNOS and HSP72 as biomarkers.

Multimarker Panel for Prognosis in Chronic Heart Failure

As a complex disease, heart failure is associated with various pathophysiological and biochemical disorders. No single biomarker is able to display all these characteristics. Therefore, a multimarker panel together with the biochemical gold-standard NT-proBNP was evaluated (Jungbauer et al. 2014). Part of the panel were biomarkers of angiogenesis (endostatin, IBP-4, IBP-7, sFlt-1 as antiangiogenetic

factors and PLGF as angiogenectic factor), myocyte stress (GDF-15), extracellular matrix remodeling (galectin-3, mimecan and TIMP-1), inflammation (galectin-3) and myocyte injury (hs-TnT). All biomarkers were positively correlated with NT-proBNP and were significantly elevated in patients with chronic HF. All markers increased significantly with severity of left ventricle dysfunction and severity of New York Heart Association class, except for PLGF and mimecan. With the exception of endostatin, mimecan and PLGF, all other biomarkers were significant predictors for all-cause mortality in a 3-year follow-up. In a multimarker approach of the five biomarkers with the best performance (NT-proBNP, hs-TnT, TIMP-1, GDF-15 and IBP-4), the event rate was superior to NT-proBNP alone and increased significantly and progressively with the number of elevated biomarkers. All emerging biomarkers increased stepwise with the severity of symptoms and left ventricle dysfunction and offer important prognostic information in chronic HF, except for PLGF and mimecan. Five biomarkers with different pathophysiological background incorporated additive prognostic value in heart failure. Thus determination of prognosis in heart failure can be further improved through a multimarker approach.

Molecular Signature Analysis in Management of Cardiovascular Diseases

In cardiovascular disorders, microarray studies have largely focused on gene expression, identifying differentially expressed genes characteristic of diverse disease states, through which novel genetic pathways and potential therapeutic targets may be elucidated. However, gene expression profiling may also be used to identify a pattern of genes (a molecular signature) that serves as a biomarker for clinically relevant parameters. Molecular signature analysis (MSA) accurately predicts the etiologic basis of heart failure and cardiac transplant rejection. These early studies provide valuable proof of concept for future work using MSA. The ultimate potential application of transcriptome-based MSA is individualization of the management of patients with structural heart disease, arrhythmias and heart failure. A patient with a newly diagnosed cardiac disease could, through molecular signature analysis, be offered an accurate assessment of prognosis and how individualized medical therapy could affect the outcome.

Presage ST2 Assay

Presage® ST2 Assay (Critical Diagnostics) measures the level of soluble ST2 in blood, identifying patients at increased risk of morbidity and mortality from cardiovascular diseases. ST2 has been evaluated in multiple clinical studies, now spanning more than 31,000 subjects. ST2 signals the presence of adverse cardiac

remodeling and fibrosis, which occur in response to MI, ischemia, or worsening heart failure. Remodeling and fibrosis can also contribute to the development of future adverse events, such as secondary MI or sudden cardiac death and to progression of HF. In the Controlled Rosuvastatin Multinational Trial in Heart Failure (CORONA study), soluble ST2 was associated with adverse outcomes in older patients with HF, and was independently associated with worsening of HF (Broch et al. 2012). Presage ST2 Assay helps clinicians assess patient prognosis in order to personalize their care.

Role of Circulating Biomarkers and Mediators of Cardiovascular Dysfunction

Both circulating biomarkers and mediators of cardiovascular dysfunction play a role in acute illness. Some of these circulating biomarkers reflect mediator action on the peripheral vasculature, such as endothelium-derived endothelin and nitrite/nitritate, the stable end products of nitric oxide (NO). Other biomarkers mainly reflect actions on the heart, such as the natriuretic peptide family, released from the heart upon dilatation, serving as a marker of congestive heart failure. Some factors may be both markers as well as mediators of cardiovascular dysfunction of the acutely ill and bear prognostic significance. Assessing circulating levels may help refine clinical judgment of the cardiovascular derangements encountered at the bedside, together with clinical signs and hemodynamic variables. For instance, assessing natriuretic peptides in patients with pulmonary edema of unclear origin may help to diagnose congestive heart failure and cardiogenic pulmonary edema, when the pulmonary capillary wedge pressure is not measured or inconclusive. Future aligning of hemodynamic abnormalities with patterns of circulating cardiovascular markers/mediators may help to stratify patients for inclusion in studies to assess the causes, response to therapy and prognosis of cardiovascular derangements in the acutely ill patients.

Use of Protein Biomarkers for Monitoring Acute Coronary Syndromes

Although it is easy to diagnose myocardial infarction, no accurate non-invasive efficient method of detecting acute coronary disease in an emergency or outpatient setting in patients with minimal or non-specific symptoms is available as yet. Many of these patients are discharged without further investigations. They are considered to be low risk as only 2–5% of patients who develop myocardial infarction later on, initially present in this manner and are discharged to home. However, more than 5% of patients who present with atypical chest pain initially are ultimately diagnosed as

acute coronary syndrome. A blood biomarker test would be useful to sort out these patients. Although a number of protein biomarkers of inflammation have been discovered, their use in outpatient setting has not been investigated adequately. Only CRP has been studied sufficiently for analysis of data. However, the threshold for a positive CRP remains unknown. Published evidence is not yet sufficient to support the routine use of new protein biomarkers in screening for ACS in the emergency department setting.

In spite of the limitation, the the most frequently used biomarker the emergency department (ED) continues to be cardiac troponin (Tn). Other biomarkers that have been used because of the need in the ED for rapid triage have been myoglobin and FABP. In addition, some centers still prefer less sensitive and less specific markers such as CK-MB. There is a trend to develop biomarkers of ischemia, such as ischemia modified albumin (IMA), to determine which patients have ischemia, even in the absence of cardiac injury. Although useful, biomarkers of ischemia are not ready for routine use. Before describing the recommendations for clinical use of biomarkers in the ED, a basic understanding of some of the science and measurement issues related to these analytes is helpful.

To explore the diagnostic accuracies of anti-apolipoprotein A-1 (anti-ApoA-1) IgG and anti-phosphorylcholine (anti-PC) IgM alone, expressed as a ratio (anti-ApoA-1 IgG/anti-PC IgM), and combined with the TIMI score for non-ST-segment elevation MI (NSTEMI) (NSTEMI-TIMI score), a new diagnostic algorithm, the Clinical Autoantibody Ratio (CABR) score, was created for the diagnosis of NSTEMI and subsequent cTnI elevation in patients with acute chest pain (Keller et al. 2012). CABR score displayed adequate predictive accuracies and could be a useful measure to rule out NSTEMI in patients presenting with acute chest pain at the ED without ECG changes.

Use of Biomarkers for Prognosis of Recurrent Atrial Fibrillation

Plasma concentrations of three specific cardiac hsTnT, NT-proBNP and mid-regional proANP (MR-proANP) and three stable fragments of vasoactive peptides (mid-regional proadrenomedullin (MR-proADM), copeptin (CT-proAVP) and CT-proendothelin-1 (CT-proET1), were measured at baseline and after 6 and 12 months in patients enrolled in the GISSI-AF (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico- atrial fibrillation) study, a prospective randomized trial to determine the effect of valsartan to reduce the recurrence of AF (Latini et al. 2011). Despite low baseline levels, higher concentrations of hsTnT, MR-proANP, NT-proBNP, CT-proET independently predicted higher risk of a first recurrence of AF. It was concluded that circulating biomarkers of cardiomyocyte injury are related to recurrence of AF in patients in sinus rhythm with a history of recent AF.

Use of Multiple Biomarkers for Monitoring of Cardiovascular Disease

Currently, screening for cardiovascular disease is primarily accomplished through a series of blood tests that determine levels of a few biomarkers. Methods of cardiovascular disease diagnosis are based on the fact that cardiac marker proteins are released into the blood in large quantities at different rates during cardiac injuries. Traditional methods, such as ELISA, can only measure one cardiac marker at a time. Nanocheck™ AMI (Nano-Ditech Corporation) provides qualitative measurements of three different well-known cardiac makers, cTnI, CK-MB and myoglobin, simultaneously or separately.

Few investigations have evaluated the incremental usefulness of multiple biomarkers from distinct biologic pathways for predicting the risk of cardiovascular events. The following biomarkers most strongly predicted the risk of death: BNP level, CRP level, the urinary albumin-to-creatinine ratio, homocysteine level, and renin level. The biomarkers that most strongly predicted major cardiovascular events were BNP level and the urinary albumin-to-creatinine ratio. It was concluded that for assessing risk in individual persons, the use of the ten contemporary biomarkers that were studied adds only moderately to standard risk factors. Because the novel biomarkers do not meaningfully reduce misclassification by traditional risk scoring, they should not be used in basic risk-factor assessment. However, the current data do not rule out a role for biomarkers such as CRP in improving risk prediction in selected patients. Although these biomarkers made a statistically significant contribution to risk prediction but that they had limited value for risk stratification of individual patients. The appropriate role of these biomarkers in the clinical care of patients remains to be determined.

Use of Biomarkers in the Management of Peripheral Arterial Disease

Peripheral arterial disease (PAD), a manifestation of systemic atherosclerosis, causes intermittent claudication with leg pain on walking or exertion. Patients with PAD have some of the other risk factors of cardiovascular disease such as inflammation, atherosclerosis and hypercholesterolemia with substantial risk of cardiovascular morbidity and death. The pentraxin family, including high-sensitivity CRP (hs-CRP), serum amyloid P (SAP), and pentraxin 3 (PTX3), has been identified as key players in inflammatory reactions such as in atherosclerosis and cardiovascular disease. CRP has been investigated as a biomarker of PAD, but could not be validated in clinical trials. A study has demonstrated that PTX3 is a better biomarker of PAD than hs-CRP or SAP, and it might be a prognostic biomarker for evaluating the severity of PAD (Igari et al. 2016).

A proteomics approach has used 2D–DIGE and MALDI-TOF MS to compare the differential plasma proteome between good and poor prognosis of PAD (Yang et al. 2017). The study identified plasma biomarkers for the screening and detection of good/poor prognosis of PAD. Among these, transthyretin and complement factor B are potential biomarkers for monitoring the PAD disease in the plasma.

Use of Biomarkers in the Management of Hypertension

Traditionally BP measurments have been considered as a marker of hypertension and response to therapy. Some of biomarkers of cardiovascular disease are now being measured as a guide to the treatment of hypertension. Diovan® (valsartan) lowered the level of the inflammatory biomarker high sensitivity C-reactive protein (hsCRP), independently of its established efficacy in lowering BP, according to a study by Novartis. The study also showed that Diovan and Co-Diovan, including two new high doses recently approved by the FDA, helped a significant number of hard-to-treat patients with moderate to severe high BP quickly achieve BP goals in as little as 2 weeks.

Systems Approach to Cardiovascular Biomarker Research

The landmark Framingham Heart Study (FHS) launched a major initiative in 2009 to discover risk factors and biomarkers that could lead to new blood tests for identifying individuals at high risk of heart disease and stroke. Called the "Systems Approach to Biomarker Research in Cardiovascular Disease (SABRe CVD)", this project will identify and validate new biomarkers such as proteins or molecules in the blood for heart disease. It is funded by NHLBI and conducted in collaboration with Boston University School of Medicine and School of Public Health. A public-private partnership has been established to enable researchers to apply cutting-edge technology to stored blood samples from thousands of FHS participants. An important component of the biomarker research will be conducted under a 5-year CRADA with BG Medicine, which has developed patented technology to detect and validate subtle biological changes at the molecular level. In 2010, Sigma-Aldrich joined this project to discover biomarkers for atherosclerosis CVD in plasma samples. Sigma will develop antibody reagents for each identified target biomarker and incorporate the reagents into a multiplexed, high-throughput platform to measure proteins of interest.

Researchers from participating institutions will study about 1000 blood biomarkers. Frozen blood samples, imaging studies, and other medical test results gathered over the years from more than 7000 FHS participants of diverse ages will be analyzed to identify which blood biomarkers are associated with heart disease, metabolic syndrome, and related risk factors. Researchers will use only materials from

participants who have consented to sharing their specimens and data with commercial sector scientists, and all shared information will be de-identified to protect participants' privacy.

The specific aims for SABRe CVD are as follows:

1. To identify the biomarker signatures of atherosclerosis as determined by: (a) aortic and coronary calcification on CT; (b) aortic plaque burden by MRI; (c) carotid intimal-medial thickness by ultrasound; (d) clinical atherosclerotic; and (e) the dynamic balance between arterial calcification and bone demineralization.
2. To identify the biomarker signatures of metabolic risk factors related to cardiovascular risk: (a) systolic and diastolic blood pressure; (b) body mass index and visceral adiposity by CT; (c) dyslipidemia; and (d) impaired fasting glucose, diabetes, and insulin resistance (glucose and insulin levels).
3. To identify genomic convergence (convergence of signals from genetic variation and gene expression) with SABRe biomarker levels and clinical traits and diseases.

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Chapter 16
Biomarkers of Pulmonary Diseases

Introduction

Lungs and airways are affected by several pathologies, the most important of which are inflammation, infection and cancer. Some of the biomarkers of these pathologies are similar to those found in involvement of other organs. This chapter will briefly discuss general issues of biomarkers of pulmonary disorders listed in Table 16.1. Biomarkers of lung cancer are described in Chapter 13.

Association of Biomarkers of Inflammation with Lung Function in the Elderly

Low lung function is associated with increased morbidity and mortality. It is therefore of interest to identify biomarkers that are associated with impaired lung function. Lung function (FEV1 and FVC) and a panel of 15 inflammatory biomarkers (including cytokines, chemokines, adhesion molecules, CRP and WBC count) from blood samples were analysed subjects aged 70 years (Kuhlmann et al. 2013). WBC count, CRP and VCAM-1 were found to relate to poorer lung function. A dose-related association was found for the combination WBC count and CRP towards FEV1 and WBC and VCAM-1 towards FVC. This indicates that combination of two biomarkers yielded more information than assessing them one by one when analysing the association between systemic inflammation and lung function.

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Table 16.1 Biomarkers of pulmonary diseases

Biomarkers	Sample	Applications
Alpha1-antitrypsin/AAT gene polymorphism	Blood: finger prick	Detection of AAT deficiency predisposing to emphysema
Angiogenic growth factor overexpression	Bronchoalveolar lavage fluid	Overexpression of VEGF and PIGF expression is a biomarker of COPD
Brain natriuretic peptide (BNP)	Plasma	Detection of pulmonary hypertension in patients with chronic lung disease
Calprotectin	Sputum and serum	Track changes in lung inflammation during an exacerbation of cystic fibrosis
CF-specific serum proteomic signature	Plasma	Cystic fibrosis (CF)
Chromagranin A (CgA)	Serum	A neuroendocrine activity biomarker that is increased in male smokers with impaired lung function.
Copeptin, the precursor of vasopressin	Serum	A prognostic biomarker for poor prognosis in exacerbation of COPD requiring hospitalization.
C-reactive protein(CRP)	Serum	Elevated in acute exacerbtion of COPD
H_2O_2 F2-isoprostanes Malondialdehyde 4-hydroxy-2-nonenal Antioxidants	Exhaled breath condensate	Measurement of oxidative stress in pulmonary diseases
Hyperuricemia	Serum	Biomarker of early mortality in COPD
IgE level	Serum	The dose of omalizumab is that required is to reduce circulating free IgE levels to less than 10 IU per milliliter
Inflammation	Blood	WBC count, CRP and VCAM-1 relate to poorer lung function in the elderly
Nitric oxide (NO)	Exhaled breath	Inflammatory lung disorders, e.g., asthma Rhinosinusitis
	Urine	Higher levels of urinary NO are strongly associated with improved survival in acute respiratory distress syndrome
Osteoprotegerin (OPG)	Serum	Increased specifically in COPD
Parathyroid hormone	Serum	Biomarker of COPD (Park et al. 2015)
Serum amyloid A (SAA)	Serum	Exacerbtion of COPD by respiratory tract infections.
Surfactant proteins: A (SP-A) D (SP-D)	Tracheal aspirates Bronchoalveolar lavage Pleural effusions	Interstitial lung disease Acute respiratory distress syndrome Radiation pneumonitis

Biomarkers of Oxidative Stress in Lung Diseases

Oxidative stress is the hallmark of various chronic inflammatory lung diseases. Increased concentrations of ROS in the lungs of such patients are reflected by elevated concentrations of oxidative stress markers in the breath, airways, lung tissue and blood. Traditionally, the measurement of these biomarkers has involved invasive procedures to procure the samples or to examine the affected compartments, to the patient's discomfort. Non-invasive approaches to measure oxidative stress have been investigated. The collection of exhaled breath condensate (EBC) is a non-invasive sampling method for real-time analysis and evaluation of oxidative stress biomarkers in the lower respiratory tract airways. The biomarkers of oxidative stress such as H_2O_2, F2-isoprostanes, malondialdehyde, 4-hydroxy-2-nonenal, antioxidants, glutathione and nitrosative stress such as nitrate/nitrite and nitrosated species can be measured in EBC. Oxidative stress biomarkers also have been measured for various antioxidants in disease prognosis. EBC is currently used as a research and diagnostic tool in free radical research, yielding information on redox disturbance and the degree and type of inflammation in the lung. It is expected that EBC can be exploited to detect specific levels of biomarkers and monitor disease severity in response to treatment.

Biomarkers of Community-acquired Pneumonia

Community-acquired pneumonia (CAP) is one of the most common reasons for emergency department. Despite its prevalence, there are many challenges to proper diagnosis and management of pneumonia. There is no accurate and timely gold standard to differentiate bacterial from viral disease, and there are limitations in precise risk stratification of patients to ensure appropriate site-of-care decisions. Clinical risk scores such as pneumonia severity index (PSI) and CURB-65 (confusion, urea, respiratory rate, blood pressure, age > 65 years), and blood biomarkers of different physiopathological pathways are used in predicting long-term survival in patients with CAP. In a prospective study, patients admitted with CAP were followed for 6 years and Cox regression models as well as area under the receiver operating characteristics curve (AUC) were used to investigate associations between initial risk assessment and all-cause mortality (Alan et al. 2015). Initial PSI and CURB-65 scores both had excellent long-term prognostic accuracy, with a step-wise increase in mortality per risk class. The addition of inflammatory (pro-adrenomedullin) and cardiac (pro-atrial natriuretic peptide) blood biomarkers measured upon hospital admission further improved the prognostic capabilities of the PSI.

Biomarkers of Acute Lung Injury and Respiratory Distress Syndrome

Cytokine/Chemokine Biomarkers of SARS

Pathological changes in severe acute respiratory syndrome (SARS) suggest that SARS sequelae are associated with dysregulation of cytokine and chemokine production. A study from Taiwan showed that cytokine or chemokine profiles in patients with SARS differ markedly from those in patients with community-acquired pneumonia (CAP) and control groups (Chien et al. 2006). Serum levels of three cytokines were significantly elevated in SARS patients versus the CAP: Interferon-γ-inducible protein-10 (IP-10), interleukin (IL)-2, and IL-6. Cytokine levels began to rise before the development of chest involvement and peaked earlier than did lung injury assessed by chest x-ray. Conversely, in CAP patients but not SARS patients or controls, levels of interferon-γ, IL-10, and IL-8 were elevated, and rose in tandem with radiographic changes. A further difference between groups was the ratio of IL-6 to IL-10, at 4.84 in SARS patients versus 2.95 in CAP patients. However, in both sets of patients, levels of IL-6 correlated strongly with the severity of lung injury. The early induction of IP-10 and IL-2, as well as the subsequent overproduction of IL-6 and lack of IL-10, probably contribute to the main immunopathological processes involved in SARS lung injury and may be early biomarkers of lung injury. These findings differ from those observed in subjects with CAP.

Plasma Biomarkers Related to Inflammation

Plasma biomarkers related to inflammation – IL-8 and enhanced neutrophil recruitment to the lung (ICAM-1) – are independently associated with increased mortality in patients with ALI. Higher levels of IL-8 and ICAM-1 independently predicted death (McClintock et al. 2008). In addition, lower levels of the coagulation marker protein C were independently associated with an increased risk of death. The association of lower protein C levels with non-survivors continues to support the role for disordered coagulation in ALI/ARDS. These associations exist despite consistent use of lung protective ventilation and persist even when controlling for clinical factors that also impact upon outcomes. The two biomarkers with an independent association with mortality, IL-8 and ICAM-1, need to be be studied further for their potential value in stratifying patients in clinical trials.

Urinary NO as Biomarker

Acute respiratory distress syndrome (ARDS) is the rapid onset of respiratory failure – the inability to adequately oxygenate the blood – that often occurs in the critically ill. Acute lung injury (ALI) precedes ARDS as severe respiratory illnesses

progress. Both conditions can be life-threatening. In a large-scale, multicenter trial of patients with ARDS or ALI, higher levels of nitric oxide (NO) in urine were strongly associated with improved survival, more ventilator-free days, and decreased rates of organ failure (McClintock et al. 2007). The authors speculated that NO has a beneficial effect on ALI since it scavenges oxygen free radicals that are generated during oxidative stress. Since NO increases microcirculation, it helps to better perfuse tissue beds in the lungs. The investigators offered an alternative hypothesis to explain their findings: NO created inside the body may have a beneficial effect on organs other than the lung during ALI. It might help prevent further tissue damage by improving oxygen and nutrient delivery to the tissues, while helping to decrease the amount of toxic oxygen species. The authors also speculated that NO might have antibacterial effects that could be important in infectious conditions that predispose patients to ALI.

Biomarkers of Interstitial Lung Disease

Pulmonary Surfactant Proteins as Biomarkers for Lung Diseases

Pulmonary surfactant, a complex of lipids and proteins, functions to keep alveoli from collapsing at expiration. Surfactant proteins A (SP-A) and D (SP-D) belong to the collectin family and play pivotal roles in the innate immunity of the lung. Pulmonary collectins directly bind with broad specificities to a variety of microorganism and possess antimicrobial effects. These proteins also exhibit both inflammatory and antiinflammatory functions. The collectins enhance phagocytosis of microbes by macrophages through opsonic and/or non-opsonic activities. The proteins stimulate cell surface expression of phagocytic receptors including scavenger receptor A and mannose receptor. Since the expression of SP-A and SP-D is abundant and restricted within the lung, the proteins are now clinically used as biomarkers for lung diseases. The levels of SP-A and SP-D in bronchoalveolar lavage fluids, amniotic fluids, tracheal aspirates and pleural effusions reflect alterations in alveolar compartments and epithelium, and lung maturity. The determination of SP-A and SP-D in sera is a noninvasive and useful tool for understanding some pathological changes of the lung in the diseases, including pulmonary fibrosis, collagen vascular diseases complicated with interstitial lung disease, pulmonary alveolar proteinosis, acute respiratory distress syndrome and radiation pneumonitis (Takahashi et al. 2006).

Serum KL-6 as Biomarker of Interstitial Lung Disease

Interstitial lung disease (ILD) is defined as restrictive lung function impairment with radiographic signs of ILD. KL-6, a mucinous high-molecular weight glycoprotein, is expressed on type II pneumonocytes and is a potential biomarker of ILD.

A retrospective, cross-sectional analysis Caucasian patients with polymyositis (PM) or dermatomyositis (DM) and ILD were shown to have elevated serum levels of KL-6 compared to patients without ILD (Fathi et al. 2012). At a cut-off level of 549 U/ml, the sensitivity and specificity for diagnosis of ILD was 83% and 100%, respectively. The level of serum KL-6 may serve as measure of ILD in patients with PM/DM, and is a promising biomarker for use in clinical practice to assess response to treatment.

Biomarkers of Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) consists of two main forms – chronic bronchitis and emphysema – and sufferers usually have a combination of these conditions. There has been increasing interest in using pulmonary biomarkers to understand and monitor the inflammation in the respiratory tract of patients with COPD. Bronchial biopsies and bronchoalveolar lavage provide valuable information about inflammatory cells and mediators, but these procedures are invasive, so that repeated measurements are limited. Sputum provides considerable information about the inflammatory process, including mediators and proteinases in COPD, but samples usually represent proximal airways and may not reflect inflammatory processes in distal bronchi. Analysis of exhaled breath is a noninvasive procedure so that repeated measurements are possible, but the variability is high for some assays. There is relatively little information about how any of these biomarkers relate to other clinical outcomes, such as progression of the disease, severity of disease, clinical subtypes or response to therapy. More information is also needed about the variability in these measurements. In the future pulmonary biomarkers may be useful in predicting disease progression, indicating disease instability and in predicting response to current therapies and novel therapies, many of which are now in development.

The COPD Foundation Biomarker Qualification Consortium (CBQC) is a unique public-private partnership established in 2010 between the COPD Foundation, the pharmaceutical industry, and academic COPD experts with advisors from the US National Heart Lung & Blood Institute and FDA (Miller et al. 2016). The initial intent of the CBQC was to integrate data collected in 2009 and submit a dossier for the qualification. This led to the FDA qualification of plasma fibrinogen as a prognostic or enrichment biomarker for all-cause mortality and COPD exacerbations in 2015. It is the first biomarker drug development tool qualified for use in COPD under the FDA's drug development tool qualification program.

Alpha1-antitrypsin Gene Polymorphisms Predisposing to Emphysema

Alpha1-antitrypsin (AAT) is a plasma glycoprotein that inhibits neutrophil elastase, and individuals who inherit altered AAT genes resulting in deficiency of the protein are at high risk for COPD and liver cirrhosis. This deficiency can be

detected by serum protein pattern studies. In the past, testing for the deficiency has been done retrospectively in patients with COPD or liver disease, but the introduction of a home-administered finger-stick blood spot test for AAT genotype enables affected families to construct pedigrees to enable them to identify children who are at risk for developing COPD in later life and should avoid exposure to dust and smoke.

Biomarkers of Extracellular Matrix Turnover in COPD

Extracellular matrix (ECM) remodeling of the lung tissue releases protein fragments into the blood, where they may be detected as serologic surrogate biomarkers of disease activity in COPD. Association of ECM turnover with severity and outcome of COPD has been assessed in a prospective, observational, multicenter study, Global Initiative for Chronic Obstructive Lung Disease grades II to IV, and serum samples were analyzed at stable state, during exacerbation as well as 4 weeks after exacerbation (Stolz et al. 2017). Results showed that patients with the lowest levels of pro-forms of collagen type III (Pro-C3) and type VI (Pro-C6) had more severe airflow limitation, hyperinflation, air trapping, and emphysema. Collagen type III (C3M) and collagen type VI (C6M) were associated with dyspnea. In conclusion, serum biomarkers of ECM turnover were significantly associated with disease severity and clinically relevant outcomes in patients with COPD.

Lung ECM remodeling in healthy controls and COPD patients was investigated in the COPDGene study. The data suggest that type VI collagen turnover and elastin degradation by neutrophil elastase are associated with COPD-induced inflammation (eosinophil-bronchitis) and emphysema (Bihlet et al. 2017). Serological assessment of type VI collagen and elastin turnover may assist in identification of phenotypes likely to be associated with progression and amenable to precision medicine for clinical trials.

Biomarkers of Lung Failure in COPD

Lung failure, also termed “lung attack”, is the most common organ failure seen in the intensive care unit. Lung attacks, which effect individuals with COPD are among the leading cause of visits to emergency rooms among chronic disease sufferers. Other causes are neuromuscular impairment, pulmonary edema, pneumonia, and vascular diseases such as acute or chronic pulmonary embolism. When a patient is admitted into the hospital with a severe lung failure, it usually takes >3 months to get to 80% of his or her baseline health. If the patient’s health is poor to start with, the new attack can be devastating or even fatal. A test that could more accurately present a patient’s disease could make it easier to predict and treat COPD progression to lung failure. There is need for a test that could be performed in any clinical lab and could be used far more widely than the current lung function tests, which are performed in certain centers by specially trained personnel.

In 2012, Canada's Prevention of Organ Failure (PROOF) Center of Excellence in Vancouver received funding from Genome British Columbia to develop a biomarker-based test for determining a COPD patient's risk for having a lung attack. Genes and protein biomarker sets that have been discovered at PROOF Center could have the ability to predict COPD-caused lung attacks and need to be validated.

BNP as a Biomarker of Chronic Pulmonary Disease

Circulating BNP levels were evaluated as a parameter for the presence and severity of pulmonary hypertension (PH) in patients with chronic lung disease (Leuchte et al. 2006). During a follow-up time of approximately 1 year, significant pulmonary hypertension (mean pulmonary artery pressure > 35 mm Hg) was diagnosed in more than one-fourth of patients and led to decreased exercise tolerance and life expectancy. Elevated BNP concentrations identified significant pulmonary hypertension with a sensitivity of 0.85 and specificity of 0.88 and predicted mortality. Moreover, BNP served as a risk factor of death independent of lung functional impairment or hypoxemia. It is concluded that plasma BNP facilitates noninvasive detection of significant PH with high accuracy and can be used as a screening test for the presence of PH. In addition, BNP enables an assessment of the relevance of PH and could serve as a useful prognostic parameter in chronic lung disease.

Chromagranin A (CgA) as Biomarker of Airway Obstruction in Smokers

A study has revealed that serum levels of the neuroendocrine activity biomarker chromagranin A (CgA) are increased in male smokers with impaired lung function, and are associated with both respiratory symptoms and the degree of airway obstruction (Sorhaug et al. 2006). The subgroup of airway epithelial cells belonging to the diffuse neuroendocrine system, termed pulmonary neuroendocrine cells, may represent a putative regulatory function of CgA as a prohormone. They are considered to control growth and development of the fetal lung and regulation of ventilation and circulation, but may also have a role in the pathogenesis of smoking-induced airway disease. The findings indicate that neuroendocrine activation may be important in smoking-related airway inflammation and remodeling, and raise the possibility that CgA could be of predictive value as a biomarker of prognosis in smoking-associated diseases.

C-reactive Protein as a Biomarker of COPD

Measurements of C-reactive protein (CRP), a biomarker of inflammation, provide incremental prognostic information beyond that achieved by traditional biomarkers in patients with mild to moderate COPD, and may enable more accurate detection of patients at a high risk of mortality. Lung function decline is significantly related

to CRP levels, with an average predicted change in FEV1 of −0.93% in the highest and 0.43% in the lowest quintile. However, respiratory causes of mortality are not significantly related to CRP levels.

Gene Expression Profile in Peripheral Blood of Patients with COPD

Genome-wide expression profiling of peripheral blood samples from subjects with significant airflow obstruction was performed to find non-invasive gene expression biomarkers for COPD (Bhattacharya et al. 2011). Correlation of gene expression with lung function measurements identified a set of 86 genes. A total of 16 biomarkers showed evidence of significant correlation with quantitative traits and differential expression between cases and controls. Further comparison of these peripheral gene expression biomarkers with those previously identified from lung tissue of the same cohort revealed that two genes, RP9 and NAPE-PLD, were decreased in COPD cases compared to controls in both lung tissue and blood. These results contribute to our understanding of gene expression changes in the peripheral blood of patients with COPD and may provide insight into potential mechanisms involved in the disease.

Hyperuricemia as a Biomarker of Early Mortality in COPD

Patients with COPD are often at high risk of early death and identification of prognostic biomarkers may aid in improving their survival by providing early intensive therapy for high-risk patients. A study has investigated the prognostic role of hyperuricemia at baseline on the prognosis of patients with COPD by retrospective evaluation of data (Zhang et al. 2015). Hyperuricemia was found to be not associated with other baseline characteristics in patients with COPD. Kaplan-Meier survival curve showed that patients with COPD with hyperuricemia had higher risk of mortality compared with patients with normouricemia. Thus, hyperuricemia is a promising biomarker of early mortality in patients with COPD.

Increased Expression of PIGF as a Biomarker of COPD

Decreased expression of vascular endothelial growth factor (VEGF) and its receptor has been implicated in the pathogenesis of COPD. Levels of placenta growth factor (PlGF), another angiogenic factor, are increased in the serum and bronchoalveolar lavage (BAL) fluid of patients with COPD and are inversely correlated with FEV1 (Cheng et al. 2008). Serum levels of PlGF in patients with COPD were more than double those in smokers and nonsmokers without COPD. These findings suggest that bronchial epithelial cells can express PlGF, which may contribute to the pathogenesis of COPD. Both PlGF and VEGF expression levels were increased in cultured bronchial epithelial cells exposed to

pro-inflammatory cytokines such as TNFα and IL-8. Although the mechanisms underlying the observed detrimental effects of PlGF remain to be clarified, persistent PlGF expression might have adverse effects on lung parenchyma by down-regulating angiogenesis.

Biomarkers of Asthma

Although the aim of management of patients with asthma is to control their symptoms and prevent exacerbations and morbidity of the disease, optimal management may require assessment and monitoring of biomarkers, i.e., objective measures of lung dysfunction and inflammation.

Biomarker for Rhinovirus-induced Asthma Exacerbation

Clinical observations suggest that rhinovirus infection induces a specific inflammatory response in predisposed individuals that results in worsened asthmatic symptoms and increased airway inflammation. A study has shown that IFN-γ-induced protein (IP)-10 is specifically released in acute virus-induced asthma, and can be measured in the serum to predict a viral trigger of acute exacerbations (Wark et al. 2007). Primary bronchial epithelial cell models of rhinovirus infection were used to identify mediators of rhinovirus infection and responded to infection with rhinovirus-16 by releasing high levels of IP-10, RANTES, and IL-16, as well as smaller amounts of IL-8 and TNF-α. IP-10, perhaps in combination with TNF-α, might be a useful clinical marker to identify rhinovirus and other virus-induced acute asthma. Additional findings suggest that IP-10 or CXCR3 (an IP-10 receptor that is highly expressed in activated T cells) might have a role in worsening of airflow obstruction and airway inflammation, and may therefore be potential therapeutic targets.

Biomarkers for Predicting Response to Corticosteroid Therapy

International guidelines on the management of asthma support the early introduction of corticosteroids to control symptoms and to improve lung function by reducing airway inflammation. However, not all individuals respond to corticosteroids to the same extent and it would be an desirable to be able to predict the response to corticosteroid treatment. Several biomarkers have been assessed following treatment with corticosteroids including measures of lung function, peripheral blood and sputum indices of inflammation, exhaled gases and breath condensates. The most widely examined measures in predicting a response to corticosteroids are airway hyperresponsiveness, exhaled NO (eNO) and induced sputum. Of these, sputum

eosinophilia has been demonstrated to be the best predictor of a short-term response to corticosteroids. More importantly, directing treatment at normalizing the sputum eosinophil count can substantially reduce severe exacerbations. The widespread utilization of sputum induction is hampered because the procedure is relatively labor intensive. The measurement of eNO is simpler, but incorporating the assessment of NO in an asthma management strategy has not led to a reduction in exacerbation rates. The challenge now is to either simplify the measurement of a sputum eosinophilia or to identify another inflammatory marker with a similar efficacy as the sputum eosinophil count in predicting both the short- and long-term responses to corticosteroids.

Comparison of Biomarkers of Asthma and COPD

Airway inflammation is associated with an increased expression and release of inflammatory reactants that regulate processes of cell migration, activation and degranulation. One study was done to quantify bronchial lavage (BAL) fluid and serum levels of IL-8, secretory leukocyte protease inhibitor (SLPI), soluble intracellular adhesion molecules-1 (sICAM-1) and sCD14, as surrogate markers of inflammatory and immune response in asthma and COPD patients with similar disease duration time (Hollander et al. 2007). Biomarkers were measured using commercially available ELISA kits. The findings show that of four measured biomarkers, only the BAL IL-8 was higher in COPD patients when compared to asthma.

Cytokines as Biomarkers of Asthma Severity

Severe asthma is characterized by elevated levels of proinflammatory cytokines and neutrophilic inflammation in the airways. Blood cytokines, biomarkers of systemic inflammation, may be a feature of increased inflammation in severe asthma. One study found that IL-8 and TNF-α levels were higher in severe asthmatics than in mild-moderate asthmatics or in controls and, in conjunction with augmented circulating neutrophils, suggest the involvement of neutrophil-derived cytokine pattern (Silvestri et al. 2006). Furthermore, in patients with severe asthma, TNF-α levels were positively correlated with both exhaled nitric oxide and circulating neutrophil counts. Cytokine levels were elevated even though the patients were on high-dose inhaled steroids. This finding might reflect the inability of these drugs to significantly suppress production of this cytokine by airway cellular sources including epithelial cells and inflammatory cells. In patients with severe asthma there may be an imbalance between IL-8 production and the blocking capacity of IL-8 autoantibodies. The findings of this study may be clinically relevant and suggest that drugs that block TNF-α release or activity might represent a new treatment option in severe asthma.

Exaled NO as a Biomarker of Asthma

Airway hyperresponsiveness is the main feature of asthma and is defined as an increase in the ease and degree of airway narrowing in response to brochoconstrictor stimuli. Inflammation plays a central role in the pathogenesis of asthma and much of it can be attributed to helper T cell type 2 cytokine activation, the degree of which strongly correlates to disease severity. One of the inflammatory mediators in asthma is nitric oxide (NO). The exhaled NO level is elevated in asthma, particularly allergic asthma during the pollen season, and can predict asthma exacerbation. It may be clinically more useful to compare exhaled NO values with a subject's previous values than to compare them with a population based normal range.

Cough variant asthma (CVA) and atopic cough both present with bronchodilator-resistant non-productive cough but may be differentiated from and other causes of chronic non-productive cough by measuring exhaled NO. Exhaled NO levels in patients with atopic cough are significantly lower than those in patients with CVA and bronchial asthma (Fujimura et al. 2008). There are no significant difference in the exhaled NO levels between patients with CVA and bronchial asthma.

A UK study findings show that it is feasible to measure bronchial flux NO concentration (^{J}NO) and alveolar NO concentration (C_{alv}) in 70% of children, with C_{alv} levels potentially reflecting alveolar inflammation in asthma (Paraskakis et al. 2006). C_{alv} and ^{J}NO were measured from the fractional exhaled NO ($FeNO_{50}$) at multiple exhalation flow rates in asthmatic children. Although $FeNO_{50}$ and JNO give essentially the same information, C_{alv} is higher in asthmatic children than in normal children. This study also highlights the relationship between poor control of asthma and C_{alv} (a biomarker of alveolar inflammation) but further work is needed to confirm the relevance of this. A novel nanosensor can detect a possible asthma attack before it begins. The minute sensor can be fitted into a hand-held device, and when a person blows into the device, it measures the NO content of their breath. Use of this device would provide asthma sufferers with a simple and cost effective way to monitor their asthma inflammation.

An explanation for increased levels of exhaled NO is nonenzymatic generation of NO from nitrite due to airway acidification in asthmatics. Reduced arginine availability may also contribute to lung injury by promoting formation of cytotoxic radicals such as peroxynitrite. As arginine levels decline, nitric oxide synthase (NOS) itself can begin to generate superoxide in lieu of NO, thereby favoring NO consumption via the generation of peroxynitrite that could induce lung injury. This reduction in bioavailability of NO via formation of species such as peroxynitrite could be further amplified by the rapid loss of SOD activity during the asthmatic response.

Plasma arginase activity declines significantly with treatment and improvement of symptoms. Additional studies are needed to determine whether measurements of plasma arginase activity will provide a useful biomarker for underlying metabolic disorder and efficacy of treatment for this disease. The arginase activity present in

serum probably does not accurately reflect whole body arginase activity or that compartmentalized in the lungs, since the arginases are intracellular enzymes. Because arginase is induced in monocytes in response to helper T cell type 2 cytokines, it is speculated that these cells are one likely source of the elevated arginase in serum, consistent with the localization of arginase expression within macrophages in the lungs.

Athough exhaled NO is a clinically useful biomarker of eosinophilic airway inflammation in asthma, significant validation and investigation are required before exhaled breath condensate could be utilized for making decisions in clinical practice (Simpson and Wark 2008).

Endothelin-1 in Exhaled Breath as Biomarker of Asthma

Endothelins are proinflammatory, profibrotic, broncho- and vasoconstrictive peptides, which play an important role in the development of airway inflammation and remodeling in asthma. A study has evaluated the endothelin-1 (ET-1) levels in exhaled breath condensate (EBC) of asthmatics with different degree in asthma severity (Zietkowski et al. 2008). ET-1 concentrations in EBC of all asthmatic patients were significantly higher than in healthy volunteers. ET-1 levels were significantly higher in patients with unstable asthma than in the two groups with stable disease. Thus, measurements of ET-1 in EBC may provide another useful diagnostic tool for detecting and monitoring inflammation in patients with asthma. The release of ET-1 from bronchial epithelium through the influence of many inflammatory cells essential in asthma and interactions with other cytokines, may play an important role in increase of airway inflammation, which is observed after postexercise bronchoconstriction in asthmatic patients.

IgE as Guide to Dosing of Omalizumab for Asthma

IgE plays a central role in the pathophysiology of asthma. The two essential phases in this pathophysiology are sensitization to allergen and clinical expression of symptoms on reexposure to the sensitizing allergen. Omalizumab (Xolair, Genentech) is a recombinant humanized IgG1 monoclonal anti-IgE antibody that binds to circulating IgE, regardless of allergen specificity, forming small, biologically inert IgE–anti-IgE complexes without activating the complement cascade. An 89–99% reduction in free serum IgE (i.e., IgE not bound to omalizumab) occurs soon after the administration of omalizumab, and low levels persist throughout treatment with appropriate doses. A total serum IgE level should be measured in all patients who are being considered for treatment with omalizumab, because the dose of omalizumab is determined on the basis of the IgE level and body weight. The dose is based on the estimated amount of the drug that is required to reduce circulating free IgE levels to less than 10 IU per milliliter.

Periostin as a Biomarker for Treatment of Asthma with Lebrikizumab

Lebrikizumab (Roche) is an injectable humanized MAb designed to block IL-13, which contributes to key features of asthma. Lebrikizumab improves lung function in adult asthma patients who are unable to control their disease on inhaled corticosteroids. IL-13 induces bronchial epithelial cells to secrete periostin, a matricellular protein. Increased levels of periostin, a biomarker of asthma, can be measured in the blood. In the MILLY phase II trial, patients with high pretreatment periostin levels had greater improvement in lung function when treated with lebrikizumab, compared to patients with low periostin levels (Corren et al. 2011).The primary endpoint of the trial showed that at week 12, lebrikizumab-treated patients had a 5.5% greater increase in lung function from the baseline compared to placebo. Lebrikizumab-treated patients in the high-periostin subgroup experienced an 8.2% relative increase from baseline forced expiratory volume in 1 second (FEV1), compared with placebo. In the low-periostin subgroup, those patients on the drug experienced a 1.6% relative increase in FEV1, compared with placebo. These results support further investigation of lebrikizumab as a personalized medicine for patients who suffer from moderate to severe uncontrolled asthma periostin enables selection of patients who will benefit most from the drug.

Biomarkers of Cystic Fibrosis

Cystic fibrosis (CF) is the most common serious genetic disease among Caucasians in the US. The disease results from a defective gene that affects multiple aspects of cellular function. Its most serious symptom is a build-up of thick, sticky mucus in the airways, which can lead to fatal lung infections. The usual method for screening and diagnosis is genotyping of cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations.

Antibody microarrays have been developed as a platform for identifying a CF-specific serum proteomic signature. Serum samples from CF patients have been pooled and compared with equivalent pools of control sera in order to identify patterns of protein expression unique to CF. The set of significantly differentially expressed proteins is enriched in protein mediators of inflammation from the NFkappaB signaling pathway, and in proteins that may be selectively expressed in CF-affected tissues such as lung and intestine. In several instances, the data from the antibody microarrays can be validated by quantitative analysis with Reverse Capture Protein Microarrays. In conclusion, antibody microarray technology is sensitive, quantitative, and robust, and can be useful as a proteomic platform to discriminate between sera from CF and control patients.

Saliva, because of the noninvasive collection process, shows great potential as a biological fluid for CF monitoring. Extensive protein degradation and differentially expressed proteins have been identified in sputum as biomarkers of inflammation relating to pulmonary exacerbations of CF. Use of fiber microarrays for measuring

significant variations of the levels of six proteins in saliva supernatants – VEGF, MMP-9, IP-10, IL-8, IL-1β and EGF – as well as the correlations of these levels with clinical assessments, has demonstrated the value of saliva for CF research and monitoring (Nie et al. 2015).

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Chapter 17
Biomarkers in Gynecology and Obstetrics

Introduction

Biomarkers relevant to women's health have not been investigated as widely as in some other therapeutic areas. Those relevant to cancer of breast and ovaries are discussed in the chapter on cancer biomarkers. This section will deal with biomarkers of menopause, endometriosis and disorders associated with pregnancy.

Biomarkers of Menopause

Menopause occurs on average at age of 51 in western societies. However, about 15% percent of women experience it early, under the age of 45. It is important to be able to predict the age of onset of natural menopause with its preceding infertility as there is a trend in delaying pregnancy. A test or a biomarker would help in making informed decisions about when to try starting to have children. Also if premature menopause could be predicted in young women, strategies to reduce the long term health risks of early estrogen deficiency could be implemented.

Changes in concentrations of estradiol, progesterone, luteinizing hormone (LH) and activin, as well as follicle dynamics occur in the endocrine regulation of ovarian function with advancing age, but they are not clinically useful for predictive testing. Other features are more promise. Among these are chronological age, family history, anti-Müllerian hormone (AMH), poor response to in vitro fertilization (IVF), basal follicle-stimulating hormone (FSH) and the antral follicle count for long term prediction (Lambalk et al. 2009). However, none of these biomarkers has been found to have sufficient predictive accuracy in individual women. Results of new and ongoing longitudinal studies may provide better predictive models. In particular, use of genetic profiles may add to the accuracy of currently known biomarkers.

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One study found that AMH, an endocrine marker that reflects the transition of resting primordial follicles to growing follicles, declined to a time point 5 years prior to the final menstrual period (FMP); this may represent a critical biological juncture in the menopause transition (Sowers et al. 2008). Low and nondetectable levels inhibin B levels also were observed 4–5 years prior to the FMP but were less predictive of time to FMP or age at FMP.

A simple blood test based on measuring levels of AMH produced by the ovaries of a young women is claimed to predict the precise age at which she will no longer be fertile. The test, based on a mathematical model using data from a large number of women, is being launched but would need to be validated in large-scale trials before it could be introduced into common use.

Biomarkers of Premenstrual Dysphoric Disorder

Premenstrual dysphoric disorder PMDD is a severe form of premenstrual syndrome that affects 5% of menstruating women. Symptoms of the disorder include marked depression, anxiety, tension, irritability and moodiness. Considered a mood disorder, women affected by PMDD have a significantly reduced quality of life. While triggered by reproductive hormones, the cause of PMDD is unknown, and current treatment options range from nutritional supplements to prescription medicine. PMDD afflicts a significant portion of the female population, yet little is known about it, and it has no definitive cure other than menopause.

Metabolon scientists will compare samples from women with PMDD to samples from normal, healthy women under controlled hormonal conditions. Metabolon will analyze the data to identify biomarkers that indicate a metabolic difference between the two groups. Results from this study could potentially lead to more effective treatments for the disorder. Metabolon's technology will identify biomarkers that can be used to develop new treatments for the disorder itself, not just the symptoms.

Biomarkers of Endometriosis

Endometriosis is a painful, chronic disease that occurs when endometrium (tissue that lines the uterus) is found outside the uterus, usually in the abdomen on the ovaries, fallopian tubes, and ligaments that support the uterus; the area between the vagina and rectum; the outer surface of the uterus; and the lining of the pelvic cavity. It affects over 5 million females in North America and millions more worldwide. This misplaced tissue develops into growths or lesions which respond to the menstrual cycle in the same way that the tissue of the uterine lining does: each month the tissue builds up, breaks down, and sheds. This results in internal bleeding, breakdown of the blood and tissue from the lesions, and inflammation. It can cause pain, infertility, scar tissue formation, adhesions, and bowel problems.

Diagnosis of endometriosis is surgical through laparoscopy, which is invasive, costly and associated with potential complications. A non-invasive test for diagnosis of endometriosis will focus the use of laparoscopy on women who are highly suspected of having endometriosis. Considerable efforts are being made to discover biomarkers of endometriosis for non-invasive diagnosis. One study has used DNA microarrays to look for biomarkers for endometriosis in peripheral blood lymphocytes in premenopausal women with or without endometriosis undergoing gynecological procedures (Flores et al. 2006). A gene selection program identified two genes, IL2RG and LOXL1, which were differentially expressed in peripheral blood lymphocytes of samples from endometriosis patients. These may provide important clues regarding the pathogenesis of this disease. Moreover, they could be considered potential targets for noninvasive diagnostic assays for endometriosis but need to be validated in a larger population. Interleukin-6 provides a promising serum biomarker for the nonsurgical prediction of endometriosis (Othman et al. 2008). Although some of the biomarkers that were investigated showed a good specificity, none of them had high sensitivity. More multicenter studies on larger numbers of patients are required to identify the most useful biomarker.

Biomarkers for Preeclampsia

Preeclampsia is an idiopathic multisystem disorder specific to human pregnancy occurring after the 20th week of gestation. There is a sudden and dangerous rise in blood pressure that can result in premature delivery, disability or death for mother and fetus. The condition, which affects 5–8% of pregnancies worldwide, constitutes a medical emergency and often requires a Cesarean section delivery. The condition is estimated to cause 50,000– 76,000 maternal deaths each year. Global health care costs for preeclampsia are estimated to be $3 billion per year. Current methods for identification of preeclampsia – regular measurements of blood pressure and protein testing in the urine during routine prenatal visits – deliver diagnosis of preeclapsia after the condition has reached an advanced status. Currently no predictive test exists for preeclampsia. Routine laboratory tests such as liver function, proteinuria and platelet count are neither accurate nor sensitive. Gene polymorphisms are being studied. Several studies have investigated protein biomarkers of preeclampsia.

Pathogenesis of Preeclampsia

Delivery of the placenta results in resolution of the condition, implicating the placenta as a central culprit in the pathogenesis of preeclampsia. In preeclampsia, an inadequate placental trophoblast invasion of the maternal uterine spiral arteries results in poor placental perfusion, leading to placental ischemia. This could result in release of factors into the maternal circulation that cause widespread activation or

dysfunction of the maternal endothelium. Factors in the maternal circulation might induce oxidative stress and/or elicit an inflammatory response in the maternal endothelium, resulting in the altered expression of several genes involved in the regulation of vascular tone.

A study found that both the TNF-α/IL-10 and sFlt-1 (fms-like tyrosine-kinase-1)/PlGF (placental growth factor) ratios were higher in placental homogenate of early-onset PE than late-onset preeclampsia and control groups (Weel et al. 2016). The more severe lesions and the imbalance between TNF-α/IL-10 and PlGF/sFlt-1 in placentas from early-onset preeclampsia enables differentiation of early and late-onset preeclampsia and suggests higher placental impairment in early-onset preeclampsia.

Women with chronic kidney disease and chronic hypertension frequently develop superimposed preeclampsia, but distinction from pre-existing disease is challenging. According to one study, plasma placental growth factor concentrations could help guide clinical decision making regarding admission and delivery for superimposed pre-eclampsia (Bramham et al. 2016).

Metabolomic Biomarkers in Urine in Preeclampsia

Metabolomic profiles, obtained in early pregnancy by NMR spectroscopy of serum and urine samples from women at medium to high risk of preeclampsia showed the following results (Austdal et al. 2015):

- Urinary metabolomic profiles predicted preeclampsia and gestational hypertension at 51.3% and 40% sensitivity, respectively, at 10% false positive rate, with hippurate as the most important metabolite biomarker for the prediction.
- Serum metabolomic profiles predicted preeclampsia and gestational hypertension at 15% and 33% sensitivity, respectively, with increased lipid levels and an atherogenic lipid profile as most important for the prediction.
- Combining maternal characteristics with the urinary hippurate/creatinine level improved the prediction rates of preeclampsia in a logistic regression model.

These results indicate that metabolomic analysis of urine has clinically important role for prediction of preeclampsia.

Protein Biomarker of Preeclampsia in Urine

A protein biomarker in urine of pregnant women could serve as a screening/diagnostic tool for preeclampsia. Many proteins present in the serum and blood could provide a clue to preeclampsia, but only the relationship between them has diagnostic significance. An algorithm is used to calculate the ratio for the presence or absence of three specific proteins that are normally secreted by human placenta; the

ratio between two of the proteins correctly identifies women who have severe preeclampsia (Adachi et al. 2006). The proteins are vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and their soluble VEGF receptor (sFlt-1). The ratio of sFlt-1 and PlGF has a high sensitivity (88%) and specificity (100%) for identifying severe preeclampsia, and is more accurate than proteinuria alone.

A panel of biomarkers present in urine could distinguish pregnant women with preeclampsia from healthy pregnant women. Combined presence of two biomarkers, specific fragments of albumin and serpina-1, is highly characteristic for preeclampsia superimposed on chronic hypertension. Further development of a new test using these biomarkers could lead to earlier diagnosis and treatment of preeclampsia and help prevent premature births.

A urinary protein, adipsinis significantly increased, and the adipsin/creatinine ratio is closely correlated with the urinary 24-h protein in patients with preeclampsia (Wang et al. 2014). When combined with the increased diastolic blood pressure (≥90 mm Hg), the sensitivity of this biomarker is 90.3% and the specificity reaches 100% for diagnosis of preeclampsia. This urine test can be used as a POC home test for monitoring onset of preeclampsia inhigh-risk pregnant women and as a rapid test for preliminary diagnosis for emergency patients at hospitals.

Protein Biomarkers of Preeclampsia in CSF

One study used proteomic analysis of CSF to identify protein biomarkers characteristic of preeclampsia and related to its severity (Norwitz et al. 2005). Samples were subjected to proteomic analysis using surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectroscopy. A discriminative proteomic biomarker profile was extracted by applying Mass Restricted analysis, and a Preeclampsia Proteomic Biomarker (PPB) score developed based on the presence or absence of four discriminatory protein peaks in individual CSF SELDI tracings. In-gel tryptic digests, Western blot analysis, on-chip immunoassays, ELISA, and spectral analysis were used to identify the biomarkers composing the PPB score. PPB score distinguished patients with a clinical diagnosis of severe preeclampsia (sPE) from mild preeclampsia (mPE) and normotensive controls. PPB scores were unaffected by parity, magnesium seizure prophylaxis, CSF leukocyte counts, and total protein content. Proteomic identification techniques matched the discriminatory protein peaks to the alpha- and beta-hemoglobin chains. ELISA confirmed that women diagnosed clinically with sPE had significantly higher CSF hemoglobin concentrations than women with mPE or CRL. Thus proteomic analysis of CSF can accurately distinguish sPE from both mPE and normotensive controls. Patients with sPE have nanomolar amounts of free hemoglobin in their CSF. Further studies are needed to confirm these observations and determine their physiologic implications.

Protein levels showed correlations with clinical symptoms during pregnancy and postpartum. The first LC-MS/MS proteome profiling study on a unique set of CSF

samples from (severe) preeclamptic patients and normotensive pregnant women showed a clear difference between the protein profiles of CSF from patients with preeclampsia and normotensive pregnant women (van den Berg et al. 2017). The most significantly differentially abundant protein found was AMBP (alpha-1-microglobulin/bikunin precursor), a precursor of a heme-binding protein that counteracts the damaging effects of free hemoglobin, which may be related to the presence of free hemoglobin in CSF.

Protein HtrA1 as a Biomarker for Preeclampsia

Protein HtrA1 is known to be involved in programmed cell death, cell change and invasiveness, i.e., the ability of cells to invade and colonize new areas. This process can be physiological as in establishing growth of a placenta in the uterus during the first trimester. Invasion also can be pathological as in the cases of cancer.

Aberrant expression of developmentally regulated genes during placental development could affect fetal growth and contribute to preeclampsia. A study was designed to determine the expression of HtrA1 in placental tissues from control and preeclamptic pregnancies to determine the effect of HtrA1 expression in trophoblast cell migration and invasion (Ajayi et al. 2008). Higher expression of HtrA1 was detected in placental tissues collected from patients with early-onset preeclampsia, compared with those from gestational age-matched control samples. Higher expression of HtrA1 is associated with early-onset preeclampsia and may affect trophoblast cell migration and invasion.

This work is the first to link high levels of HtrA1 in third-trimester placental tissues with severe preeclampsia. Though preliminary, the findings may 1 day lead to development of a blood test to track HtrA1 levels to identify women at risk of preeclampsia. Although the initial results are really encouraging, it is too early to say if HtrA1 is a definite biomarker of preeclampsia. Because greater amounts of HtrA1 indicate greater placental distress and disease severity, developing a blood test to detect levels of HtrA1 may possibly serve as an early warning system that placental conditions are changing. The hope is that such a predictive test would allow physicians to manage preeclampsia on a nonemergency basis when it is less threatening for mother and fetus, or possibly to devise therapies to stop the process or prevent it altogether.

Placental Growth Factor as a Biomarker for Preeclampsia

Serum placental growth factor (PlGF) is a member of the VEGF sub-family and a key molecule in angiogenesis, in particular during embryogenesis. The main source of PlGF during pregnancy is the placental trophoblast. It is a first-trimester biomarker for preeclampsia. The measurement of maternal PlGF at 11–13 weeks

of gestation provides an excellent screening test both for chromosomal defects and for the diagnosis of preeclampsia. Clinical trials have shown that PlGF supports the diagnosis and risk prediction for preeclampsia. Thermo Fisher has licenseed PlGF from Nephromics will develop it as an immunoassay on its KRYPTOR™ platform.

sFlt1 and Soluble Endoglin as Biomarkers of Preeclampsia

Maternal endothelial dysfunction mediated by excess placenta-derived soluble VEGF receptor 1 (sVEGFR1) or soluble fms-like tyrosine kinase (sFlt1) is emerging as a prominent component in disease pathogenesis. Increased levels of free sFlt-1 have been measured by immunoassay from serum and urine samples of preeclampsia patients. However, for unknown reasons, a subset of preeclampsia patients will go on to experience severe preeclampsia – a group of dramatically escalated symptoms characterized by a sudden, massive rise in blood pressure, which can lead to the onset of seizures, as well as the development of fetal growth restriction and the HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome, which indicates that the mother's liver and blood-clotting systems are not functioning properly, and the health of both mother and infant are in serious danger. Investigation of HELLP led to the search for another factor that acts jointly with sFlt1 to induce vascular damage and escalate the disease to its severe form.

A novel placenta-derived soluble TGF-β co-receptor, endoglin (sEng), which is elevated in the sera of preeclamptic individuals, correlates with disease severity and falls after delivery. sEng inhibits formation of capillary tubes in vitro and induces vascular permeability and hypertension in vivo. Its effects in pregnant rats are amplified by coadministration of sFlt-1, leading to severe preeclampsia including the HELLP syndrome and restriction of fetal growth. sEng impairs binding of TGF-β to its receptors and downstream signaling including effects on activation of eNOS and vasodilation, suggesting that sEng leads to dysregulated TGF-β signaling in the vasculature. These results suggest that sEng may act in concert with sFlt-1 to induce severe preeclampsia and can be considered a biomarker of this condition. This has important diagnostic and therapeutic implications for the management of this disease

RNA Biomarkers

Placental RNA analyzed in maternal plasma permits rapid screening of novel biomarkers including biomarkers not accessible by antibody based assays. This includes transcription factors, non-coding RNA (ncRNA), epigenetic features, as well as genes functionally or genetically linked with preeclampsia. Review of genes with placental expression in the Human SymAtlas and comparison with proven

qualifiers, has revealed a large number of RNA biomarkers that may be useful for the presymptomatic detection in first trimester of pregnancy-associated diseases with placental origin and/or dysfunction such as pregnancy-induced hypertension without or with proteinuria (preeclampsia), and intrauterine growth restriction.

A laser microdissection approach has enabled the identification of novel mRNAs and ncRNAs that were misexpressed by various trophoblast subpopulations in severe preeclampsia (Gormley et al. 2017). Many of the newly identified dysregulated molecules might have clinical utility as biomarkers of severe preeclampsia.

Genes Associated with Preeclampsia

Polymorphisms of the adiponectin gene show a weak, but statistically significant, haplotype association with susceptibility to preeclampsia in Finnish women (Saarela et al. 2006). Although many genetic screens in multiple populations have been performed, only two preeclampsia susceptibility genes (ACVR2A, STOX1) have been identified within confirmed regions with significant genome-wide linkage (van Dijk and Oudejans 2011). STOX1 genotype has been shown to directly limit first trimester extravillous trophoblast invasion, the earliest hallmark of preeclampsia. The authors have described upstream regulation and downstream effector genes of the transcription factor STOX1 and proposed a model combining the cell type-specific and allele-specific effects of STOX1 including intrinsic effects (differential CpG island methylation) and extrinsic effects (regulation of effector genes).

Biomarkers of Premature Birth

It is estimated that preterm labor complicates 6–10% of all pregnancies and is the most common cause of neonatal morbidity and mortality. Worldwide statistics reflect that there may be as many as 13 million preterm births annually, and this figure is predicted to increase. In the US, an estimated $820 million is spent on preterm hospitalization subsequently shown to have been unnecessary. These hospitalizations tax both the mother's health and healthcare resources. There is a need for rapid or accurate tests that positively predict preterm labor.

Metabolome Inc. has identified specific metabolites or biomarkers in the amniotic fluid metabolome that enabled classification of patients at risk for preterm delivery. Women who present at 26 weeks of pregnancy with premature elabor have three outcomes; (1) false labor with normal full-term pregnancy; (2) premature birth; and (3) bacterial infection leading to inflammation. A stereotypic pattern of metabolites were identified in different groups. These observations enable identification of metabolic pathways which are altered in preterm labor. A third blinded study to refine the algorithms in order to improve the precision is now being planned as well as studies in urine and plasma.

Proteomic Biomarkers of Premature Birth

By profiling specific proteins in amniotic fluid for inflammation, researchers at Yale School of Medicine (New Haven, CT) can quickly and accurately detect potentially dangerous infections in pregnant women, and also predict the possibility of premature birth by Mass Restricted (MR) score as a specific proteomic profile. Presence of the biomarkers indicating inflammation in amniotic fluid can be established in 20–30 min. This test is much faster than the current method of testing microbiological cultures. If no biomarkers are present, then the pregnancy is uncomplicated. Proteins in a small sample of amniotic fluid were tested to find a link between the amniotic fluid glucose value, white blood cell count and the outcome of the fetus. An MR sore of three or four is highly predictive of adverse pregnancy outcome. The presence of two biomarkers for inflammation indicates the median time for delivery is 4 days. If all the biomarkers for inflammation are present, delivery time occurs within hours. Studies to test treatment were not possible before. The results of this test can be used to provide a rapid treatment to the mother and its baby.

PreTRM test (Sera Prognostics) is performed using LC-MS/MS to simultaneously measure multiple proteins in patients' blood. These proteins have been proven to be associated with spontaneous preterm birth. The concentrations of individual proteins are then combined to establish a PreTRM test result that represents an individual woman's risk of spontaneous preterm delivery for her current pregnancy. The test was validated to predict spontaneous preterm birth risk by measuring proteins that are over- or under-expressed and are predictive of premature birth or delivery. PreTRM test is highly predictive of preterm delivery in asymptomatic, singleton pregnancies where blood is drawn at 19–20 weeks gestational age, with reported spontaneous preterm birth prediction Area Under the Curve (AUC) ranging between 0.75 and 0.93. PreTRM is a robust method for determining relative abundances of biomarkers and enables accurate prediction of individual risk of spontaneous preterm birth (Bradford et al. 2017).

Microfluidic microchip systems with pressure-driven injection for electrophoretic analysis of amino acids, peptides, and proteins have been developed to separate biomarkers implicated in pre-term birth (Sahore et al. 2016). Although these devices were initially demonstrated as a stand-alone microfluidic separation tool, they have a strong potential to be integrated within more complex systems for diagnosis of pre-term birth.

Circulating microparticles in pregnancy are potential biomarkers because they represent an in vivo biopsy of active gestational tissues. NX Prenatal is investigating the ability of protein biomarkers extracted from exosomes to predict spontaneous preterm delivery. A study of plasma samples using MS has identified functional proteomic factors with associated biological processes that are already unique in their expression profiles at 10–12 w among women who go on to deliver spontaneously $\leq$34 weeks (Cantonwine et al. 2016). These changes, with further validation, will enable stratification of patients at risk of spontaneous preterm birth before clinical presentation.

Biomarkers of Oxidative Stress in Complicated Pregnancies

Oxidative stress may contribute to the development of complications in pregnancy and antioxidant activity in both maternal and umbilical cord blood may be an indicator of oxygen radical activity. Various parameters of oxidative stress have been measured in pregnancies with hypertension and preeclampsia, insulin dependent diabetes mellitus, gestational diabetes mellitus, oligohydramnios and abruptio placentae, as well as a healthy control group. The results of these studies suggest that oxidative stress and subsequent lipid peroxidation accompany the complications of hypertension, preeclampsia and diabetes mellitus in pregnancy. Maternal erythrocyte GST activity seems to be a sensitive indicator of oxidative stress before delivery. The same enzyme can be used in cord blood as a biomarker of oxidative stress upon a sudden increase in oxygenation during delivery. These multiparameter biomarkers can also be used in monitoring the efficiency of antioxidant supplementation in complicated pregnant women.

Low plasma antioxidant status, assessed through a Antioxidant Status (Antiox-S) score during early normal pregnancy, is associated with development of complications later on in pregnancy (Ramiro-Cortijo et al. 2016). Larger population studies are needed to determine the value of Antiox-S as predictive tool and to determine the role of nutrition on maternal antioxidant status.

Fetal Biomarkers in Maternal Blood

Cell-free fetal nucleic acids in maternal plasma or serum can be used for noninvasive prenatal diagnosis, such as the determination of fetal blood groups and fetal gender. The discovery of fetal mRNA transcripts in the maternal circulation holds great promise for noninvasive prenatal diagnosis. To identify potential fetal biomarkers, RNA was isolated from peripheral or umbilical blood and hybridized to gene expression arrays (Maron et al. 2007). Gene expression, paired Student's t test, and pathway analyses were performed. These identified fetal biomarkers included developmental genes, sensory perception genes, and genes involved in neonatal physiology. Transcripts were predominantly expressed or restricted to the fetus, the embryo, or the neonate. Real-time RT-PCR amplification confirmed the presence of specific gene transcripts; SNP analysis demonstrated the presence of fetal transcripts in maternal antepartum blood. Comparison of whole blood and plasma samples from the same pregnant woman suggested that placental genes are more easily detected in plasma. These findings show that fetal and placental mRNA circulates in the blood of pregnant women. Transcriptional analysis of maternal whole blood identifies a unique set of biologically diverse fetal biomarkers and has a multitude of clinical applications. It enables at-risk pregnancies to be identified, enabling the modification of pregnancy management and thus improvement of pregnancy outcome, but accurate quantification of fetal nucleic acids and specificity of these elevations for particular disorders remain controversial issues (Visca et al. 2011).

Metabolic Biomarkers of Prenatal Disorders in the Mother

A NMR metabonomic study of second trimester maternal urine and plasma has attempted to characterize metabolic changes underlying prenatal disorders and identify possible early biomarkers (Diaz et al. 2011). Fetal malformations have the strongest metabolic impact in both biofluids, suggesting effects due to hypoxia (leading to hypoxanthine increased excretion) and a need for enhanced gluconeogenesis, with higher ketone bodies (acetone and 3-hydroxybutyric acid) production and TCA cycle demand (suggested by glucogenic amino acids and cis-aconitate overproduction). Choline and nucleotide metabolisms also seem affected and a distinct plasma lipids profile is observed for mothers with fetuses affected by CNS malformations. Urine from women who subsequently develop gestational diabetes mellitus exhibits higher 3-hydroxyisovalerate and 2-hydroxyisobutyrate levels, probably due to altered biotin status and amino acid and/or gut metabolisms (the latter possibly related to higher BMI values). Other urinary changes suggest choline and nucleotide metabolic alterations, whereas lower plasma betaine and TMAO levels are found. Chromosomal disorders and pre-preterm delivery groups show urinary changes in choline and, in the latter case, in 2-hydroxyisobutyrate. These results show that NMR metabonomics of maternal biofluids enables the noninvasive detection of metabolic changes associated to prenatal disorders, thus unveiling potential disorder biomarkers.

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Chapter 18
Biomarkers & Personalized Medicine

Introduction

Personalized medicine, also referred to as individualized therapy, simply means the prescription of specific treatments and therapeutics best suited for an individual taking into consideration both genetic and environmental factors that influence response to therapy. Personalized medicine is described in a textbook on this topic (Jain 2015). Genomic/proteomic technologies have facilitated the development of personalized medicines but other technologies are also contributing to this effort. Personalized medicine is the best way to integrate new biotechnologies into medicine for improving the understanding of pathomechanism of diseases and management of patients. Important basics of personalized medicine are derived from the following technologies and approaches:

1. Molecular diagnostics, particularly SNP genotyping
2. Biomarkers
3. Integration of diagnostics with therapy, particularly monitoring of therapy
4. Bioinformatics for evaluation and use of data from various biotechnologies
5. Pharmacogenomics
6. Pharmacogenetics
7. Pharmacoproteomics

Development of personalized medicine is closely linked to biomarkers, which may serve as the basis for diagnosis, drug discovery and monitoring of diseases. This process of personalization starts at the development stage of a medicine and is based on pharmacogenomics, pharmacogenetics and pharmacoproteomics, which will be described in the following sections.

The economics and uncertainties of drug development demand that companies quickly demonstrate the safety and efficacy of their compounds. Using biomarkers to make key development decisions early can reduce development timelines and

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increase the likelihood of regulatory and clinical success, thereby maximizing market positioning. Biomarkers are becoming more critical in the process of discovering and developing new drugs and in determining additional uses for established drugs. Biomarkers are ushering in the age of personalized medicine.

Pharmacogenetics

Pharmacogenetics, a term recognized in pharmacology in the pregenomic era, is the study of influence of genetic factors on action of drugs as opposed to genetic causes of disease. Now it is the study of the linkage between the individual's genotype and the individual's ability to metabolize a foreign compound. The pharmacological effect of a drug depends on pharmacodynamics (interaction with the target or the site of action) and pharmacokinetics (absorption, distribution and metabolism). It also covers the influence of various factors on these processes. Drug metabolism is one of the major determinants of drug clearance and the factor that is most often responsible for interindividual differences in pharmacokinetics.

The differences in response to medications are often greater among members of a population than they are within the same person or between monozygotic twins at different times. The existence of large population differences with small intrapatient variability is consistent with inheritance as a determinant of drug response. It is estimated that genetics can account for 20 to 95% of variability in drug disposition and effects. Genetic polymorphisms in drug-metabolizing enzymes, transporters, receptors, and other drug targets have been linked to interindividual differences in the efficacy and toxicity of many medications.

Although interindividual variations in drug response result from effects of age, sex, disease or drug interactions, genetic factors represent an important influence in drug response and efficacy and remain constant throughout life. This has led to the recognition of the discipline "pharmacogenetics" since the 1950s, which can be viewed an as integration of gene profiling and pharmaceutical chemistry. From this initial definition, the scope has broadened so that it overlaps with pharmacogenomics.

Pharmacogenomics, a distinct discipline within genomics, carries on that tradition by applying the large-scale systemic approaches of genomics to understand the basic mechanisms and apply them to drug discovery and development. Pharmacogenomics now seeks to examine the way drugs act on the cells as revealed by the gene expression patterns and thus bridges the fields of medicinal chemistry and genomics. Some of the drug response markers are examples of interplay between pharmacogenomics and pharmacogenetics; both are playing an important role in the development of personalized medicines. The two terms – pharmacogenetics and pharmacogenomics – are sometimes used synonymously but one must recognize the differences between the two as shown in Table 18.1.

Table 18.1 Pharmacogenetic vs. pharmacogenomic studies

Feature	Pharmacogenetics	Pharmacogenomics
Focus of studies	Patient variability	Drug variability
Scope of studies	Study of sequence variations in genes suspected of affecting drug response	Studies encompass the whole genome
Methods of study	SNP, expression profiles and biochemistry	Gene expression profiling
Relation to drugs	One drug and many genomes (patients)	Many drugs and one genome
Examination of drug effects	Study of one drug in vivo in different patients with inherited gene variants	Examination of differential effects of several compounds on gene expression in vivo or in vitro
Prediction of drug efficacy	Moderate	High value
Prediction of drug toxicity	High value	Moderate
Application relevant to personalized medicine	Patient/disease-specific healthcare	Drug discovery and development or drug selection

Biomarkers and Pharmacogenetics

Identification and characterization of a large number of genetic polymorphisms (biomarkers) in drug metabolizing enzymes and drug transporters in an ethnically diverse group of individuals may provide substantial knowledge about the mechanisms of inter-individual differences in drug response. Pharmacogenetics is used in preclinical investigations for biomarkers of drug-response or drug-induced toxicity, identification of genes with variants that may define patient populations, identification proteins as potential biomarkers, or the comparison of the response in human and clinical animal models. Application of pharmacogenetic biomarkers should be able to predict adverse reactions in clinical trials. Role of pharmacogenetic biomarkers in personalized medicine is shown in Fig. 18.1.

Pharmacogenomics

Pharmacogenomics applies the large-scale systemic approaches of genomics to drug discovery and development. It also involves the study of the mechanisms by which drugs change the expression of genes, including drug-metabolizing enzymes, a phenomenon known as induction. Various technologies enable the analysis of these complex multifactorial situations to obtain individual genotypic

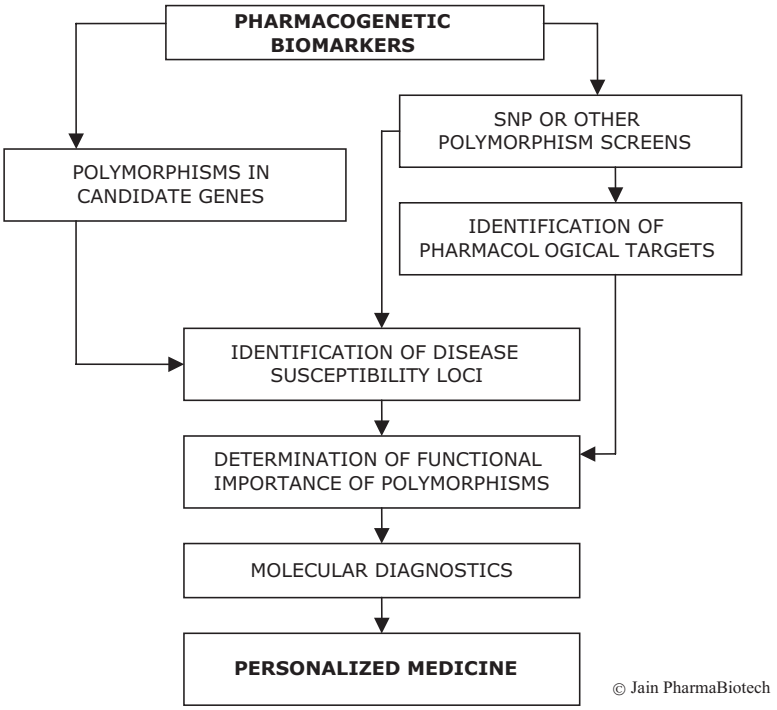

Fig. 18.1 Role of pharmacogenetic biomarkers in personalized medicine (© Jain PharmaBiotech)

and gene expression information. These same tools are applicable to study the diversity of drug effects in different populations. Pharmacogenomics promises to enable the development of safer and more effective drugs by helping to design clinical trials such that non-responders would be eliminated from the patient population and take the guesswork out of prescribing medications. It will also ensure that the right drug is given to the right person from the start. In clinical practice, doctors could test patients for specific SNPs known to be associated with non-therapeutic drug effects before prescribing in order to determine which drug regimen best fits their genetic makeup. Pharmacogenomic studies are rapidly elucidating the inherited nature of these differences in drug disposition and effects, thereby enhancing drug discovery and providing a stronger scientific basis for optimizing drug therapy on the basis of each patient's genetic constitution. Pharmacogenomics can now be redefined as the study and application of DNA-, and RNA-based biomarkers to predict how an individual's genetic inheritance affects the body's response to a drug. Pharmacogenomic biomarkers should be able to predict drug efficacy in clinical trials. Discovery and validation of pharmacogenomics biomarkers can lead to the development of pharmacogenomic tests that can be used to personalize therapy.

Pharmacoproteomics

Role of proteomics in drug development can be termed "pharmacoproteomics". Proteomics-based characterization of multifactorial diseases may help to match a particular target-based therapy to a particular marker in a subgroup of patients. The industrial sector is taking a lead in developing this area. Individualized therapy may be based on differential protein expression rather than a genetic polymorphism.

LumiCyte Inc. is building a health- and disease-specific proteomics database by collecting protein profiles on a scale not possible until now. The unique computer-based design of LumiCyte's BioChips enables the direct integration of its protein profiles with information derived from existing genomics, proteomics, and clinical databases. The fusion of these molecular and clinical databases, and the mining of the aggregated content define LumiCytes' powerful BioPhore Knowledgebase. The BioPhore Knowledgebase will be the world's first Internet-based platform of aggregated and integrated bioinformatics focused on the delivery of individualized healthcare.

Proteomics will have a great impact on diagnosis during the first decade of the twenty-first century. By the end of the decade protein chip-based tests will be available for several diseases. Knowledge gained from genomics and proteomics will be combined to provide optimal detection of disease at an early stage for prevention or early intervention. Proteomics-based molecular diagnostics will have an important role in the diagnosis of certain conditions and proteomics-based medicines would be integrated in the total healthcare of a patient. Advantages of application of pharmacoproteomics in personalized medicine are:

- Pharmacoproteomics is a more functional representation of patient-to-patient variation than that provided by genotyping.
- Because it includes the effects of post-translational modification, pharmacoproteomics connects the genotype with the phenotype.
- By classifying patients as responders and non-responders, this approach may accelerate the drug development process.

Applications of pharmacoproteomic biomarkers in personalized medicine are shown in Table 18.2.

Single Cell Proteomics for Personalized Medicine

Owing to the complexity of the intracellular metabolic pathways, an understanding of the intracellular pathways has been lagging behind the advances in gene expression. Multicolor FACS (fluorescence activated cell sorting) techniques combined with phosophospecific antibodies have been developed, enabling the determination of relative phosphorylation of signal transduction intermediates in individual cells. When stimulated with cytokines, individual leukemia cells exhibit marked differences in phosphoprotein patterns, which correspond with disease outcome. Thus, single cell phosphoproteomic techniques are superior to other proteomic

Table 18.2 Applications of pharmacoproteomic biomarkers in personalized medicine

Toxicoproteomics for prediction of toxicity during drug development
As markers of drug response and efficacy
For patient stratification in clinical trials
Protein biomarkers as common denominator of diagnostics and therapeutics
Tailoring of therapy in the postmarketing phase

technologies for the molecular diagnosis of disease and development of personalized medicine. Although study of the phosphoprotein network is usually associated with oncology, such a technology might be useful for other diseases for which multiple treatment options exist and competing technologies have not been able to adequately predict the optimal treatment for individual patients.

Role of Biomarkers in Development of Personalized Drugs

The role of biomarkers in drug development is discussed in Chap. 4. In addition to personalizing the use of existing drugs, the development of new personalized drugs should start at the discovery stage. Eexamples of this are metabolomic biomarker-based drug discovery, and pharmacogenetics/pharmacogenomics-based monoclonal antibody (MAb) drug development in oncology.

Metabolomic Biomarker-based Drug Discovery

Identification of small molecule metabolites present in patient-derived samples can support discovery of novel biomarkers, which may help in the discovery of personalized medicines individualize treatments is challenging. Biomarkers for individualization of treatment can be identified using a clinical metabolomics-based approach, and concepts used in pharmacodynamic/pharmacokinetic modeling can be integrated in this process in order to increase the clinical relevance of identified biomarkers and personalized medicine (Kohler et al. 2017). A workflow chart of this process is shown in Fig. 18.2.

Use of Biomarkers for Developing MAb Therapy in Oncology

The significance of pharmacogenomics in MAb therapeutics is highlighted by the association between polymorphisms in Fc receptors and clinical response to anti-CD20 MAb rituximab (Rituxan) or anti-ganglioside GD2 MAb 3F8, as well as the potential link between polymorphisms in HER2 and cardiac toxicity in patients treated with the anti-HER2 MAb trastuzumab (Herceptin). The dependence on

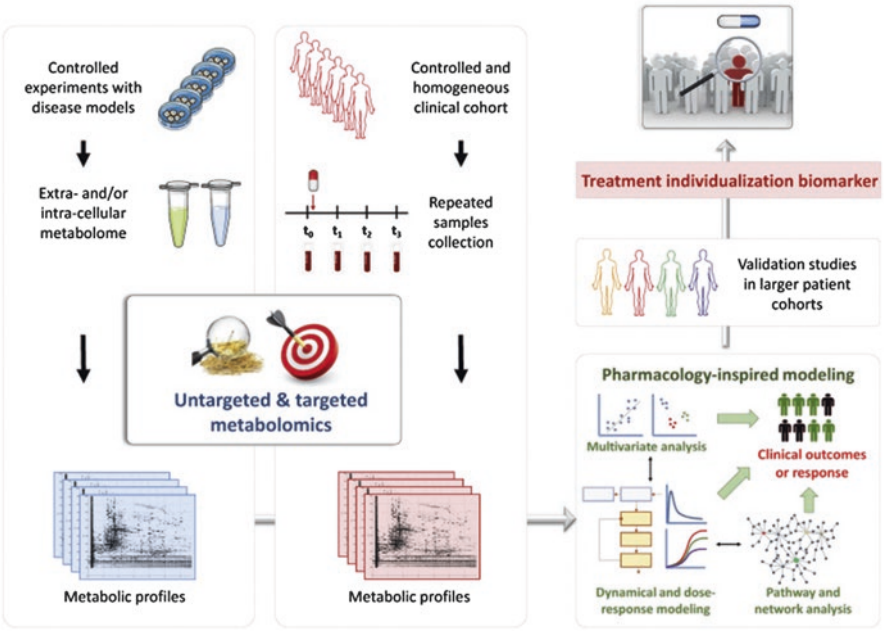

Fig. 18.2 Workflow for developing metabolomics-based biomarkers for personalized treatment (Kohler et al. 2017. Reproduced by permission of the author (copyright holder))

gene copy number or expression levels of HER2 and epidermal growth factor receptor (EGFR) for therapeutic efficacy of trastuzumab and cetuximab (Erbitux), respectively, supports the importance of selecting suitable patient populations based on their pharmacogenetic profile. In addition, a better understanding of target mutation status and biological consequences will benefit MAb development and may guide clinical development and use of these innovative therapeutics. The application of pharmacogenetics and pharmacogenomics in developing MAb therapeutics will be largely dependent on the discovery of novel surrogate biomarkers and identification of disease- and therapeutics-relevant polymorphisms. There are many opportunities as well as challenges in biomarker discovery and validation, and in implementing clinical pharmacogenetics and pharmacogenomics in oncology MAb development.

Biomarker Tests for Molecularly Targeted Therapies

A committee of the National Academy of Sciences, USA, identified 10 goals to advance the development and appropriate clinical use of biomarker tests for molecularly targeted therapies (Graig et al. 2016):

1. Establish common evidentiary standards of clinical utility — using evidence generated both within and outside the context of clinical trials — across all stakeholders.

2. Establish a more coordinated and transparent federal process for regulatory and reimbursement decisions for biomarker tests for molecularly targeted therapies.
3. Enhance communication to patients and providers about the performance characteristics and evidence for use of specific biomarker tests for molecularly targeted therapies.
4. Update and strengthen oversight and accreditation of laboratories providing biomarker tests for molecularly targeted therapies.
5. Ensure ongoing assessment of the clinical utility of biomarker tests for molecularly targeted therapies.
6. Ensure development and use of EHRs and related biomedical informatics tools and assessment that support the effective clinical use of biomarker tests for molecularly targeted therapies.
7. Develop and maintain a sustainable national database for biomarker tests for molecularly targeted therapies through biomedical informatics technology to promote rapid learning for the improvement of patient care.
8. Promote equity in access to biomarker tests for molecularly targeted therapies and the expertise for effective use of the results in clinical decision making.
9. Enhance specimen handling and documentation to ensure patient safety and the accuracy of biomarker test results.
10. Improve the processes for developing and updating clinical practice guidelines for the effective use of biomarker tests for molecularly targeted therapies.

Biobanking, Biomarkers and Personalized Medicine in EU

The Biobanking and Biomolecular Research Infrastructure (BBMRI), which started the preparatory phase in 2008, started to pool all of the major biobanks in Europe. Together these represent ~12 million blood, body fluid, and tissue samples. In the following two years, BBMRI created the preconditions to make the biological materials and data available, as well as to standardize the analyses platforms and sample preparation. The project not only includes the organization and funding of the EU biobank, but also aims to establish a complete resource for EU life scientists, including a variety of affinity binders and molecular tools as well as a biocomputing infrastructure that will work with standardized protocols, making data generated from those materials more comparable. The BBMRI was selected for FP7 funding as one of six EU infrastructure projects that are supposed to benefit all EU researchers.

No single biobank can be large enough to generate statistically significant data of specific disease subtypes and it takes more than a few dozen or even hundreds of cases in well-defined diseases to correlate disease history or patient response to a certain therapy and to biomarkers. The 134 associated partners of the BBMRI could together provide about 2.4 million samples from population-based biobanks, and a further 10 million from disease-orientated biobanks. The project will seek to overcome the current fragmentation in biobanking, and could also become an

interesting tool for the biopharmaceutical industry when validating biomarkers. The information generated from BBMRI will be useful for the development of personalized medicine.

The joint initiative, which will tie together Europe's top research groups across almost every area of molecular and cell biology, also has a political dimension. Because the protection of the data obtained from biological samples continues to be a sensitive subject, the initiative will need to conform with all the national legislations involved. For that purpose, the partners plan to establish a widely-accepted and harmonized set of practices in line with the heterogeneous landscape of European and national regulations. For instance, the protocol to be added to the Convention of Human Rights, which was approved by the EU Council in 2007 and has now been sent out to member nations for ratification, states that the confidentiality of the information obtained through diagnostic, predictive and pharmacogenetic tests of the samples must be assured. The researchers will have to find procedures that assure a high degree of data protection while simultaneously allowing use of the patient data to acquire deeper insights into the causes of disease. Three types of biobanks have been considered as source of biomarkers in EU (Riegman et al. 2008).

1. Population banks. Their primary goal is to obtain biomarkers of susceptibility and population identity, and their operational substrate is germinal-line DNA from a huge number of healthy donors, representative of a concrete country/region or ethnic cohort.
2. Disease-oriented banks for epidemiology. Their activity is focused on biomarkers of exposure, using a huge number of samples, usually following a healthy exposed cohort/case-control design, and studying germinal-line DNA or serum markers and a great amount of specifically designed and collected data.
3. Disease-oriented general biobanks (i.e. tumor banks). Their goals correspond to biomarkers of disease through prospective and/or retrospective collections of tumor and no-tumor samples and their derivates (DNA/RNA/proteins), usually associated to clinical data and sometimes associated to clinical trials. Those data are usually not collected for a concrete research project, except in case of clinical trials, but from the healthcare clinical records. The amount of clinical data linked to the sample determinate the availability and biological value of the sample.

Currently, the Biobanking and BioMolecular resources Research Infrastructure Austria (BBMRI.at) is the Austrian national node of the European biobanking research infrastructure BBMRI-ERIC. BBMRI.at consists of and links Austrian universities and biobanks with the goal to establish a national biobanking research infrastructure for accelerating biomedical research. BBMRI.at aims to establish a state-of-the-art biobanking infrastructure in Austria and to increase close cooperation and harmonization between biobanks. These are prerequisites to facilitating access and fostering the use of biological samples and data for academic and industrial research. Biologic samples and data collected in biobanks are valuable resources for innovations in personalized medicine, and development of biomarkers, diagnostics and therapeutics.

Bioinformatics to Sort Biomarker Data for Personalized Medicine

In 2012, the Center of Excellence for the Prevention of Organ Failure (PROOF) and IO Informatics started collaboration to develop a web-based software application addressing chronic heart, lung, and kidney diseases. The application will be developed so that clinicians can use it on handheld device and other technology, and it will be used with blood tests developed by the PROOF Center (http://www.proofcentre.ca/) that target chronic disease and transplantation. Its biomarker programs will be used to develop simple blood tests to improve prediction, diagnosis, management and treatment of diseases, by harnessing the power of clinical, molecular and computational science. It has biomarker programs across a multitude of disease indications.The application will give an overall score indicating patient risk level and associated clinical recommendations to help guide decision making. The scores and recommendations will be based on gene expression data, protein expression data, and longitudinal clinical observations. Future applications of the technology will enable automated, pre-symptomatic screening for biomarker-based risk events, disease severity characterization, and treatments that are suitable for individual patients.

Biomarkers for Monitoring Response to Therapy

One of the important aspects of personalized medicine is the ability to monitor response to therapy. There are some examples in various diseases mentioned in the preceding chapters. A few more examples are given here to show the value of biomarkers as well as their limitations in monitoring response to therapy.

Sensitive noninvasive strategies for monitoring treatment response in rheumatoid arthritis (RA) would be valuable for facilitating appropriate therapy and dosing, evaluating clinical outcome, and developing more effective drugs. Because different proteases are highly up-regulated in RA and contribute significantly to joint destruction, the suitability of such enzymes as in vivo imaging biomarkers for early evaluation of treatment response has been investigated in animal models of RA. Protease-activated near-infrared fluorescence (NIRF) imaging “smart” probes are sensitive means of imaging the presence of target enzymes in arthritic joints and can be used for early monitoring of treatment response to antirheumatic drugs such as methotrexate.

Assessment of hepatic damage associated with chronic hepatitis B in the past relied on measurement of serum transaminases and assessment of hepatic histology. Additionally, the liver fibrosis biomarkers type IV collagen, amino-terminal propeptide of type I procollagen (PINP), and carboxy-terminal telopeptide of type I collagen (ICTP) have been used for monitoring the effect of lamivudine therapy for chronic hepatitis B. Results showed that PINP/ITCP ratio is sensitive and specific in

detecting responders to treatment. For HBeAg (hepatitis B e antigen)-positive patients tenofovir is the most effective drug, whereas for HBeAg-negative patients, either tenofovir or entecavir is most effective (Govan et al. 2015).

Drug Rescue by Biomarker-based Personalized Medicine

Biomarkers can rescue drugs by identifying the patients that respond to them. Herceptin, initially approved in 1998, emerged as a winner only a decade after clinical trials showed little or no efficacy and is now a billion dollar blockbuster. Only when the 20% to 30% of women whose tumors overexpress HER2 were singled out was the drug's efficacy indisputable. In the pivotal clinical trial of patients with metastatic breast cancer, tumor-response rates to Herceptin plus chemotherapy were 45%, compared to 29% for chemotherapy alone.

But response is not wholly predictable. Reported response rates for HER2-positive cancers vary from less than 20% to more than 75%. HER2-positive cells that don't respond to Herceptin may have more active forms of the kinase Akt. And HER2 belongs to a receptor family that can be activated by 11 different soluble proteins and combinations thereof. Researchers are already betting that working out the biology behind the biomarker will lead to better treatments. Another anticancer antibody based on this understanding is already in clinical trials.

Similarly, the lung-cancer drug Iressa (gefitinib) could be rescued by a diagnostic based on a biomarker. Unfavorable clinical trial results dashed high hopes for big sales, but finding the patients most likely to benefit changed prospects. Various studies found that patients that responded to Iressa had mutations in the gene for EGFR.

Future Role of Biomarkers in Personalized Medicine

Personalized medicine is being recognized by the biopharmaceutical industry, regulatory authorities, healthcare providers and the medical profession. It should be a part of healthcare system by the year 2015. Genetic testing will improve predictions of disease predisposition, onset, severity as well as treatments or medications that are likely to be efficacious or harmful. Impact of biomarkers on personalized medicine is shown schematically in Fig. 18.3.

The important points of role of biomarkers in development of personalized medicine are:

- Biomarkers will enable early diagnosis of disease to facilitate optimization of therapy.
- Biomarkers will play an important role in combining diagnosis with therapeutics – an important feature of personalized medicine.

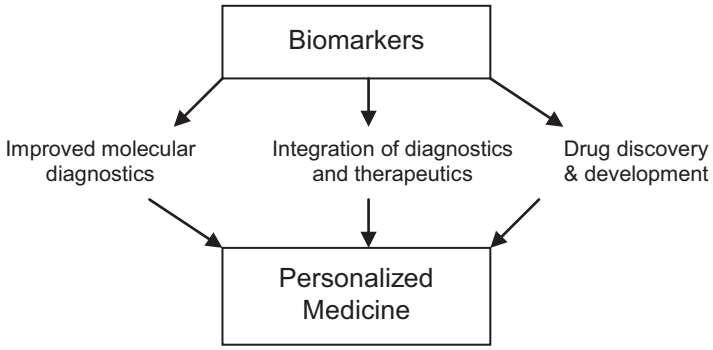

Fig. 18.3 Impact of biomarkers on personalized medicine (© Jain PharmaBiotech)

- There will be an increase in the number of new drugs suitable for personalized treatment, which will be discovered by use of biomarkers.
- Validated biomarkers will play an increasing role in clinical trials for personalizing therapeutics.
- Biomarker-based monitoring of drug efficacy will guide personalized management of several diseases.

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Chapter 19
Biomarkers and Regulatory Issues

Introduction

The diagnostic performance of a novel biomarker-based test involves the assessment of its diagnostic accuracy (sensitivity and specificity) and predictability in a series of studies from phase I (exploratory phase) to IV (outcome phase), which require different performance characteristics and different study populations. Once the clinical usefulness of a novel biomarker has been demonstrated, the decision for commercial development is made by a company. Technical, medical, legal, financial and regulatory considerations also influence this decision. A company with immunoanalyzer platform can transfer a research immunoassay into an automated procedure that meets basic laboratory requirements. The company will then evaluate the new assay and gather the required validation date for the regulatory authorities. Timelines of biomarker development are shown in Fig. 19.1.

Many of the regulatory issues concerning biomarkers are related to genomics, proteomics, molecular diagnostics and pharmacogenomics/pharmacogenetics. The quality standards for biomarker assays used in support of nonclinical safety studies fall under GLP (FDA) regulations, whereas, those assays used to support human diagnostics and healthcare are established by CLIA (CMS) regulations and accrediting organizations such as the College of American Pathologists. While most research applications of biomarkers are not regulated, applications in clinical trials require validation.

Biomarker Validation

Biomarker validation is the process of assessing the assay or measurement performance characteristics and biomarker qualification (clinical validation) is the process of providing evidence to link a biomarker with biology and clinical endpoints.

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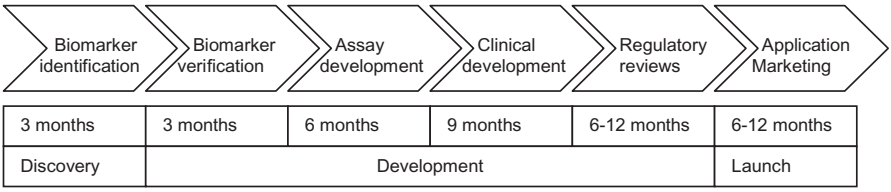

Fig. 19.1 Stages and timelines of biomarker discovery, development and marketing (© Jain PharmaBiotech)

FDA Criteria for a Valid Biomarker

Numerous criteria for the validation of the surrogacy of a biomarker have been proposed. An older concept defined two conditions that together are sufficient to ensure the surrogacy of a biomarker; (1) a strong and significant correlation between the biomarker and the clinical endpoint; and (2) the biomarker should fully capture the net effect of the treatment on the true clinical endpoint. The second condition is extremely restrictive, and represents the main reason for the failure of biomarkers to prove their surrogacy. Understanding this limitation, the FDA proposed a somewhat less restrictive criterion for considering evidence based on the use of biomarkers for drug approval purposes, where the second conditions is not reinforced if the biomarker is reasonably likely, on epidemiological, therapeutic, pathophysiological or other evidence, to predict clinical benefit. However, approval under this section is subject to the requirement that the applicant studies the drug further to verify and describe its clinical benefit in adequate and well-controlled post-marketing studies. FDA's "Guidance on Biomarker Qualification" details the formal steps involved in qualifying biomarkers for approval.

A pharmacogenomic test result may be considered a valid biomarker if it is measured in an analytical test system with well-established performance characteristics and there is an established scientific framework or body of evidence that elucidates the physiologic, pharmacologic, toxicologic, or clinical significance of the test results. For example, the effects of genetic variation in the human enzymes CYP2D6 and thiopurine methyltransferase on drug metabolism are well recognized scientifically and are included in some approved drug labels. The results of genetic tests that distinguish allelic variants of these enzymes are considered to be well established and, therefore, valid biomarkers.

A probable valid biomarker is one that is measured in an analytical test system with well-established performance characteristics and for which there is a scientific framework or body of evidence that appears to elucidate the physiologic, toxicologic, pharmacologic, or clinical significance of the test results. A probable valid biomarker may not have reached the status of a known valid marker because, for example, of any one of the following reasons:

- The data elucidating its significance may have been generated within a single company and may not be available for public scientific scrutiny.

- The data elucidating its significance, although highly suggestive, may not be conclusive.
- Independent verification of the results may not have occurred.

The distinction between what tests are appropriate for regulatory decision making and those that are not will change over time as the science evolves. Throughout the development of these tests, as appropriate, FDA will continue to seek public comment as we evaluate whether a biomarker is a valid biomarker (e.g. via discussions at Advisory Committee meetings).

Algorithms described in the FDA Pharmacogenomics Guide for investigational and marketing application holders describe when to submit to FDA data on known valid biomarkers. Data on probable valid biomarkers need not be submitted to the IND unless they are used by a sponsor to make decisions regarding specific animal safety studies or clinical trials (e.g. using biomarker data as inclusion or exclusion criteria, assessment of treatment-related prognosis, or stratifying patients by dose) or are a probable valid biomarker in human safety studies. However, FDA recommends that sponsors or applicants submit reports on all probable valid biomarkers to new (i.e. unapproved) NDAs or BLAs according to the algorithm. Many pharmacogenomic testing programs implemented by pharmaceutical sponsors or by scientific organizations are intended to develop the knowledge base necessary to establish the validity of new genomic biomarkers. During such a period of scientific exploration, test results are not useful in making regulatory judgments pertaining to the safety or effectiveness of a drug and are not considered known or probable valid biomarkers. Biomarker qualification pilot process at the FDA is shown in Fig. 19.2.

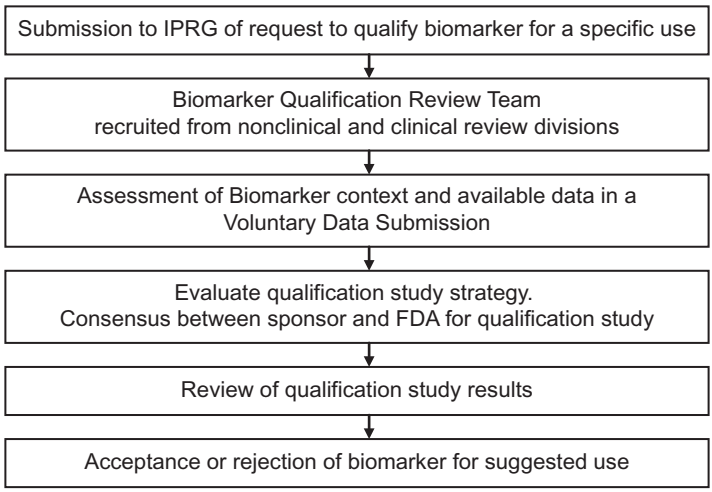

Fig. 19.2 Biomarker qualification pilot process at the FDA. Abbr: *IPRG* Interdisciplinary Pharmacogenomics Review Group (Source: FDA as published in Goodsaid et al. 2008)

FDA Letter of Support for Biomarkers

To encourage further development of promising biomarkers, which are not yet ready for qualification, FDA may issue a Letter of Support to submitters who have assembled this information about promising biomarkers. This letter briefly describes CDER's thoughts on the potential value of a biomarker and encourages further evaluation. This letter does not connote qualification of a biomarker. It is meant to enhance the visibility of the biomarker, encourage data sharing, and stimulate additional studies. Further details are given on FDA's web site. Some issued letters of support by FDA are shown in Table 19.1.

Role of NIST in Validation of Cancer Biomarkers

Rigorous validation of biomarkers for early detection of cancer differs at the National Institute of Standards and Technology (NIST) from similar processes common among research laboratories. As a newly discovered biomarker assay

Table 19.1 Issued letters of support for biomarkers by the FDA

Submitter	Biomarkers	Area(s) for further evaluation
Critical Path (C-Path) Institute's Predictive Safety Testing Consortium (PSTC), Nephrotoxicity Working Group	Urinary biomarkers: osteopontin and neutrophil gelatinase-associated lipocalin	Early clinical drug development
C-Path, PSTC, Skeletal Muscle Working Group	Serum and plasma biomarkers: myosin light chain 3, skeletal muscle troponin I, fatty acid binding protein 3, muscle type creatine kinase	Early clinical drug development
C-Path, Coalition Against Major Diseases (CAMD) Consortium	CSF analyte biomarkers: Aβ1–42, total tau, phosphotau	Exploratory prognostic biomarkers for enrichment in early stage Alzheimer disease clinical trials
C-Path, CAMD	MRI biomarker: low baseline hippocampal volume	Exploratory prognostic biomarkers for enrichment in early stage Alzheimer disease clinical trials
C-Path, CAMD	Molecular neuroimaging biomarker: dopamine transporter	Exploratory prognostic biomarkers for enrichment in early stage Parkinson disease clinical trials
C-Path, Polycystic Kidney Disease Outcomes Consortium	MRI, CT, or ultrasound biomarker: total kidney volume	Exploratory prognostic biomarker for enrichment in autosomal dominant polycystic kidney

Source: FDA

makes the transition from a research setting to the clinical diagnostic laboratory, it should progress through defined stages of assay confirmation. The NIST Cancer Biomarker Validation and Reference Laboratory assesses cost, efficiency and reliability of potential diagnostic techniques using biomarkers before further clinical evaluation in other laboratories of the NCI's Early Detection Research Network (EDRN). The first task of this laboratory is evaluation of research assay technology, performance, and specifications (analytical validation). However, the ultimate goal is initial validation of the test to identify early stage cancer (clinical validation). Upon technical and clinical confirmation, assays are moved systematically toward a standardized, reproducible, high-throughput format for clinical diagnostic implementation. With laboratory performance rigorously established, the clinical variables can subsequently be analyzed to define limitations, applications, and clinical utility. The role of NIST in technology evaluation for early cancer testing is described in the context of similar programs and prior experience at NIST (http://nist.gov/). The laboratory conducted the full mitochondrial genome sequencing using capillary electrophoresis and detected mtDNA changes associated with early cancer (see Chap. 13). Validation steps of cancer test development at NIST impact health care through institutional focus on measurement, technology, and standards development programs.

Quality Specifications for BNP and NT-proBNP as Cardiac Biomarker Assays

The Committee on Standardization of Markers of Cardiac Damage of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) published quality specifications for BNP and NT-proBNP as cardiac biomarkers assays in 2005. The recommendations proposed were intended for use by manufacturers of commercial assays, by clinical laboratories using those assays, by clinical trial groups and research investigators, and by regulatory agencies, such as the FDA. The main objectives of these recommendations were as follows:

- The manufacturers should endorse and then consistently follow the proposed recommendations.
- All package inserts for B-type natriuretic peptide immunoassays should include uniform information on assay design, preanalytical performance characteristics, analytical performance characteristics, and clinical performance characteristics.
- Quality specifications for each assay should be published in peer-reviewed journals.
- Regulatory agencies should adopt a minimal and uniform set of criteria for manufacturers to provide when seeking clearance for new and/or improved assays.

National Biomarker Development Alliance

The National Biomarker Development Alliance (NBDA, http://nbdabiomarkers.org/) is a non-profit organization launched in 2014 that is engaging leaders in industry, academia, patient groups and government to address the complex and urgent challenge of creating the standards needed to support end-to-end evidence-based biomarker development in order to significantly advance personalized health care get more biomarkers on the market. According to the NBDA, 150,000 papers have documented protein biomarker "discoveries," but only ~100 biomarkers are in clinical use these days. NBDA will achieve its goals through a management construct and systems-based approach that integrates and leverages biomarker knowledge networks from all of these stakeholder communities. It will help to create rules such as best practices and standard operating procedures for biomarker development and will share information about biomarkers among stakeholders, including patient groups, researchers, and physicians. The group will develop a database of standards for genomics, proteomics, imaging, and other biomarkers, and will convene a meeting to get a consensus among the community.

FDA Perspective of Biomarkers in Clinical Trials

Although the current regulations permit the FDA to base the approval of a drug on a determination the effect of the drug on an unvalidated surrogate biomarker, i.e., one for which it is not known that an effect on the surrogate actually predicts the desired clinical benefit. There are a number of difficulties in interpreting trials that use surrogate biomarkers as primary measures of drug effect. The primary difference between a biomarker and a surrogate biomarker is that a biomarker is a "candidate" surrogate biomarker, whereas a surrogate biomarker is a test used, and taken, as a measure of the effects of a specific treatment. There are regulatory problems related to the interpretation of clinical trials in which unvalidated surrogate biomarkers are used as primary outcomes.

When a clinical benefit can clearly be shown in reasonable clinical trials, the FDA will not rely on an effect on a surrogate to support approval. The regulations impose numerous requirements on sponsors of drug products approved under these provisions, e.g., the regulations apply only to proposed treatments for serious and life-threatening conditions that "…provide meaningful therapeutic benefit… over existing treatments…," they require that studies be done after marketing to demonstrate the link between the effect on the surrogate and the predicted clinical benefit, and they require that the Agency evaluate the advertising materials of a sponsor before their use. Underlying these requirements is the recognition that approval based on the effect of a drug on an unvalidated surrogate marker introduces uncertainty about the drug's true clinical benefit that is ordinarily not present at the time of approval under more typical circumstances. The regulations are meant

to apply only in those clinical situations in which this level of uncertainty is acceptable but, even then, only temporarily; that is, while attempts are ongoing to "validate" the surrogate. If the attempt to validate the surrogate fails (or no reasonable attempt is made), the drug may be removed from the market.

The FDA has approved drug products on the basis of their effects on surrogate markers for many years before the adoption of current regulations (examples include anti-hypertensives, cholesterol-lowering agents, and treatments for glaucoma). In each of these cases, drugs have been shown to have a beneficial effect on the surrogate of interest (blood pressure, serum cholesterol, and intra-ocular pressure, respectively) without a requirement that they be shown to have an effect on the relevant, desired clinical outcomes (heart attacks, strokes, death in the first two cases, and visual loss in the last). What distinguishes these cases from the cases to which the new regulations and law are intended to apply is that in the former cases, there is (or presumed to be) evidence that the effect on the surrogate does, in fact, predict the desired clinical outcome; that is, these are considered "validated" surrogate markers.

Clinical trials designed to demonstrate lack of disease progression in conditions such neurodegenerative disorders are difficult. Now that many new investigational treatments designed to affect the underlying pathology of various diseases have appearance, there is renewed interest in relying on the effect of a drug on surrogate markers to support approval. For example, effects of a treatment for Alzheimer's disease (AD) on total brain volume as assessed by MRI is a biomarker. Before one could interpret any difference between treatment and control on this measurement as being beneficial to patients, several questions would need to be satisfactorily addressed. First, one would need to be assured that there is no interaction between the test system, e.g. MRI, and the treatment, giving rise to a completely spurious relationship between treatment and apparent benefit. That is, such an interaction could exist (similar to the interactions known to occur when the presence of one drug interferes with the assay for a second drug in plasma). Assuming that such an interaction can be excluded, the question of how to interpret any actual structural change as seen on MRI must be addressed. It is possible that, in fact, the drug does induce a structural change in the brain, as seen on MRI, but that the change is clearly irrelevant to any presumed beneficial effect of the treatment. For example, an increase in extracellular fluid might be interpreted as a decrease in brain atrophy (or an increase in brain volume), but this would clearly not be a structural change of interest.

Because knowledge of the relevant pharmacologic and biologic events is always imperfect and incomplete, drugs are typically approved on the simple finding of a beneficial effect in adequate clinical trials (and on adequate safety data). This is ideal, not only because it is a direct clinical benefit that is obviously desired by the patient, but also because waiting to fully understand the relevant biologic events before drugs are approved is an undesirable strategy.

This is why approval of a drug on the basis of an effect on an unvalidated surrogate marker represents such a fundamental departure from the typical course of action in drug approval. That is, approval of a drug on the basis of such an effect

presupposes knowledge of events that is normally not only absent, but, in a sense, irrelevant. Therefore, approval based on effects on surrogate markers will invariably involve a level of uncertainty not typical of the more standard route to drug approval.

An effect on a surrogate marker can be proposed as predicting the desired clinical benefit of a specific treatment. Also as noted above, in these cases, regulations require that sponsors "validate" the marker after approval has been granted. Typically, this "validation" takes the form of demonstrating that there is, in fact, a strong correlation between the effect on the surrogate and the clinical outcome of interest (e.g., decreased mortality). Such a showing is ordinarily taken to provide evidence that the effect on the surrogate predicted the effect on the clinical outcome.

However, surrogate markers are most useful when they can be more generically "validated." That is, instead of validating the surrogate for a specific drug, the ideal approach would be for a sponsor (more realistically, this would require the cooperation of the entire therapeutic community) to demonstrate that the effect on the surrogate and the effect on the clinical outcome are quantitatively similar across many drugs (and especially across many drug classes designed to treat a given clinical indication). In this way, one could be confident (although still not absolutely certain) that an effect seen on the surrogate for the next (as yet untested) proposed treatment will have the expected and desired clinical effect. In other words, such an approach is the only reliable method of demonstrating (although, again, not with certainty) that the effect seen on the surrogate, regardless of the treatment applied, will translate into the clinical benefit. This is the case currently for anti-hypertensives and cholesterol-lowering agents.

FDA and Predictive Medicine

The FDA released a white paper in 2004 entitled "Innovation or Stagnation? Challenge and Opportunity on the Critical Path to New Medical Products" (http://www.fda.gov/oc/initiatives/criticalpath/whitepaper.html). This white paper was a serious attempt by the FDA to bring attention and focus to the need for targeted scientific efforts to modernize the tools, techniques and methods used to evaluate the safety, efficacy and quality of drug products. It described the urgent need for cooperation between the FDA, the NIH and the private sector to modernize the development process for medical products - the Critical Path - to make product development more predictable and less costly. The critical path determines the potential bottlenecks in bringing a product to market. The focus of the Critical Path Initiative is to identify ways to update the product development infrastructure for drugs, biologics and devices, and the evaluative tools currently used to assess the safety and efficacy of new medical products. Examples of evaluative tools include the use and verification of pathophysiological and/or descriptive biomarkers for patient selection for clinical trials and/or use as surrogate endpoints. In addition, an important example of a scientific opportunity for improving the critical path is the

use of pharmacogenomics and pharmacogenetics or, more specifically, the identification of DNA-based biomarkers or RNA-expression profiles that can provide insights into the stage of a disease, disease progression, drug response and drug-dosing requirements, and thereby lead to the development of tests to predict clinical outcomes more reliably (Lesko and Woodcock 2004).

Biomarkers and FDA's Voluntary Genomic Data Submission

In 2006, FDA had already more than 2 years of experience in its Voluntary Genomic Data Submission (VGDS) program and it suggested that the program be expanded to include proteomic, metabolomic, and other types of biomarker data. The FDA is open-minded in reviewing proteomic biomarkers. There were 25 data submissions in VGDS program and the rate of VGDS submissions – about one per month – has remained stable. With one exception, none of the data submitted under the voluntary program has been resubmitted as part of a required review. Given the length of drug development process, the 2-year life of the program is relatively short, so that no conclusions can be drawn as yet. Recent submissions have been trending toward the preclinical area. Top therapeutic areas include cancer, Alzheimer's disease, and depression. Top technologies include genotyping, microarrays, and software validation.

Role of Imaging Biomarkers in Approval of Drugs

Imaging can frequently provide crucial supportive data for regulatory approval. Etanercept, e.g., is a tumor necrosis factor (TNF) inhibitor for the treatment of rheumatoid arthritis. When examining the potential for etanercept as a first-line treatment, early trials used two sets of criteria: (i) American College of Rheumatology (ACR) scores, which use a combination of subjective pain and function assessments, in addition to serum C-reactive protein levels; and (ii) conventional radiography images of joint-space narrowing and erosion. Whereas clinical scoring showed no significant difference between etanercept and methotrexate (the standard therapy at the time), the imaging-based erosion score showed statistically significant differences. On the basis of these data, the FDA granted marketing approval of etanercept with the condition that additional supporting data be collected. A subsequent study was able to show that etanercept achieved sustained improvements over methotrexate in terms of both clinical and imaging scores, thus gaining first-line treatment status.

Multiple sclerosis (MS) is a complex disease with several treatment options available in the USA, including disease-modifying agents IFN-β-1a and IFN-β-1b for treating relapsing-remitting MS. Despite these successes, clinical trials for MS raise several issues, such as ethical concerns associated with conducting long-term

placebo trials, and the size and cost of trials comparing new therapies with existing pharmaceuticals. As such, there has been greater reliance on imaging data in the conduct of these clinical trials, especially using T2-MRI lesion burdens (i.e. lesion number and load), in addition to the number of contrast-enhancing lesions. Within the US regulatory process, the FDA permits the use of biomarkers provided that such biomarkers are reasonably likely to predict future clinical outcome. As such, because of inconsistent correlations between T2 lesion burden and contrast-enhancing lesion frequency to clinical scores, these imaging biomarkers can serve as primary outcome measure in exploratory trials (i.e. phase I and phase II), but can only serve as secondary outcome in pivotal trials. Despite this reluctance to over-emphasize the importance of imaging, a recent trial with MS patients on IFN-β-1b treatment showed marked improvements in various imaging biomarkers over a 3-year period.

More than half of the anticancer drugs that received accelerated approval for marketing during the past decade did not rely upon traditional survival data in clinical trials. Surrogate endpoints were used in these trials, the most common one being the change in tumor size resulting from drug therapy as measured by imaging (MRI or CT). Thus imaging biomarkers are recognized for regulatory approval in oncology. There is one drawback of relying on the rate of tumor size changes as a biomarker as it does not always correlate with survival, indicating that other factors confound the relationship between tumor response and survival.

Regulatory Oversight of Biomarker Tests for Targeted Therapies

The current regulatory structure for biomarker tests for molecularly targeted therapies features key oversight authority by two federal agencies: the FDA and the Centers for Medicare & Medicaid Services (CMS). Numerous state regulatory bodies and professional and accreditation organizations also are involved and provide complementary oversight of diagnostic tests and laboratory operations. Biomarker tests that will be used to identify patients likely to benefit from a specific investigational targeted therapy may be co-developed with the drug; the biomarker and drug are both tested simultaneously in clinical trials, and the safety and efficacy of the test and the drug are evaluated in the same trial. Biomarker tests that are co-developed with a drug and co-approved by FDA are known as companion in vitro diagnostics (see later in this chapter).

FDA and Biomarkers

The FDA views the use of biomarker diagnostics as an important component of its new Critical Path Initiative, an agency-wide effort to ensure rapid transfer of innovative products from the research bench to the clinical bedside. The FDA is

developing guidance and refining its regulatory tool box. FDA's goal is to ensure that it works as a partner rather than an obstacle in promoting the development of cutting edge new medical products. In 2006, the FDA released a list of high-priority research projects for its Critical Path Initiative and highlighted biomarker development as one of the "most important areas for improving medical product development."

FDA Consortium Linking Genetic Biomarkers to Serious Adverse Events

In 2007, the FDA's decided to create a consortium with members of the pharmaceutical industry and academia that aims to observe how genetic biomarkers contribute to serious adverse events (SAEs). It will be part of the Office of Critical Path Programs. Some people are genetically predisposed to have SAEs to some drugs, the FDA is of the opinion that it not in its best interests or that of the drug manufacturers to simply launch these products without putting appropriate information on labels.

Some pharmaceutical companies are sceptical and and will not join as they think that the consortium will have little effect on tracking and avoiding SAEs. The problem is that it will take thousands and thousands of patients to screen in order to validate a particular marker. SAEs, which include hepatotoxicity, rabdomyalysis, and QT prolongation, among others, typically occur in less than one in 1000 patients and are inherently unpredictable either by preclinical or clinical development. It is because of the rarity of such events, the prospect of predicting them by genetic biomarkers is viewed as not only daunting but unlikely.

The Serious Adverse Events Consortium is not the only federal initiative aimed at improving drug safety. The Critical Path is also linking the Association of Clinical Research Organizations with the Clinical Data Interchange Standards Consortium to form the Clinical Data Acquisition Standards Harmonization project. This group is charged with developing sample case report forms for reporting adverse events There is cross-cutting coordination and harmonization of all the centers within the FDA. These include the Oncology Biomarker Qualification Initiative, which pairs the FDA with the National Cancer Institute and the Centers for Medicaid and Medicare Services; the Biomarker Consortium, which brings together the FDA, the NIH, and the Pharmaceutical Research & Manufacturers of America. Areas of focus in this effort are bioinformatics and data standards, biomarkers, establishing public-private partnerships, and developing guidance and regulations.

Oncology Biomarker Qualification Initiative

The Oncology Biomarker Qualification Initiative (OBQI) is an agreement between the FDA, the NCI, and the Centers for Medicare & Medicaid Services (CMS) to collaborate on improving the development of cancer therapies and the outcomes for

cancer patients through biomarker development and evaluation. The collaboration develops scientific understanding of how biomarkers can be used to assess the impact of therapies and better match therapies to patients. For instance, OBQI addresses questions such as how particular biomarkers can be used to:

- Determine if a patient's tumor is likely to respond at all to a specific treatment
- Assess after one or two treatments if a patient's tumor is responding to treatment
- Determine more definitively if a tumor is dying, even if it is not shrinking
- Identify which cancer patients are at high risk of their tumor recurring after therapy
- Efficiently evaluate whether an investigational therapy is effective for tumor

The goal of OBQI is to validate particular biomarkers so that they can be used to evaluate new, promising technologies in a manner that will shorten clinical trials, reduce the time and resources spent during the drug development process, improve the linkage between drug approval and drug coverage, and increase the safety and appropriateness of drug choices for cancer patients.

Under the OBQI, biomarker research will be focused in four key areas: (1) standardizing and evaluating imaging technologies to see in more detail how treatments are working; (2) developing scientific bases for diagnostic assays to enable personalized treatments; (3) introducing new trial designs that use biomarkers; and (4) pooling data to ensure that key lessons are shared between organizers of clinical trials. By working with academic and industry scientists, as well as professional organizations, the OBQI teams can foster the development of key information on biomarkers through clinical trials. By identifying biomarkers for specific cancers and clinically evaluating them, researchers will have an evidence base for their use in targeted drug development and to determine which therapies are likely to work for patients before treatment selection. Rather than waiting weeks to months to determine if a specific drug works for a patient, biomarkers could be used to monitor real-time treatment responses.

The first OBQI project validated and standardized PET scans used to characterize biochemical changes in a cancer. Under the collaboration, researchers use FDG-PET imaging technology in clinical trials of patients being treated for non-Hodgkin's lymphoma, to determine if FDG-PET is a predictor of tumor response. Data resulting from this type of evidence-based study will help both FDA and CMS work with drug developers based on a common understanding of the roles of these types of assessments. In the following several months, the OBQI team will design a number of initiatives to identify and clinically qualify other cancer biomarkers. This brings together scientists from many sources and address agency priorities identified through FDA's Critical Path and NIH's Roadmap Initiatives. The OBQI also represents the work of the NCI-FDA Interagency Oncology Task Force (IOTF) – collaboration between NCI and FDA to enhance the efficiency of clinical research and the scientific evaluation of new cancer treatments. The two agencies, along with CMS, share knowledge and resources to facilitate the development of new cancer drugs and diagnostics and speed their delivery to patients as safely and as cost-effectively as possible.

Critical Path Initiative

The Critical Path Institute was formed in 2005 to support the FDA's Critical Path Initiative via collaborative research programs. The Critical Path Opportunity Report, issued in 2006, is the first specific blueprint for the FDA's Critical Path Initiative, an effort to streamline the drug-approval process by applying new strategies and technologies. It can be viewed on the FDA web site: https://www.fda.gov/ScienceResearch/SpecialTopics/CriticalPathInitiative/default.htm. The report points out that a new product development toolkit – containing powerful new scientific and technical methods such as animal or computer-based predictive models, biomarkers for safety and effectiveness, and new clinical evaluation techniques – is urgently needed to improve predictability and efficiency along the critical path from laboratory concept to commercial product. In a statement announcing the priority list, the FDA said it would rely on "partnerships and consortia to accomplish a majority of the projects," as well as "a new, cooperative partnership among the primary divisions of Health and Human Services." The will identify additional specific research projects for the initiative. According to the Report, there are substantial opportunities for better diagnoses, more efficient drug development, and safer and more effective therapies, such as the identification and qualification of new safety and efficacy biomarkers. The goal of critical path research is to develop new, publicly available scientific and technical tools – including assays, standards, computer modeling techniques, biomarkers, and clinical trial endpoints – that make the development process itself more efficient and effective and more likely to result in safe products that benefit patients. These advances will play an essential role in helping industry, regulators and other stakeholders deliver on the promise of personalized medicine. Among the 76 projects outlined, biomarker development and clinical trial reform were areas cited by researchers as most likely to improve the efficiency of product development. Examples of other opportunities are:

Additional biomarkers (quantitative measures of biological effects that provide informative links between mechanism of action and clinical effectiveness) and additional surrogate markers (quantitative measures that can predict effectiveness) are needed to guide product development. In some cases, datamining and analysis, with possibly a single additional clinical trial, may be all that is necessary to confirm the surrogacy of a particular marker. In other cases (e.g., the NIH's Osteoarthritis Initiative), epidemiologic studies on disease natural history must be undertaken to provide data on markers of disease processes. For biomarkers that currently appear promising, specific projects need to be undertaken to:

- Assemble existing data on the association of the marker with clinical outcomes
- Assemble existing data on the performance of the marker during intervention trials compared to the performance of current outcome measures
- Identify any data gaps or remaining uncertainties
- Identify clinical trials under development in which the remaining questions could be addressed in a straightforward manner

Imaging technologies, such as molecular imaging tools in neuropsychiatric diseases or as measures of drug absorption and distribution, may provide powerful insights into the distribution, binding, and other biological effects of pharmaceuticals, but their predictive value needs further study and evaluation. New imaging technologies will ultimately contribute important biomarkers and surrogate endpoints, but how soon these new tools will be available for use will depend on the effort invested in developing them specifically for this purpose.

Strengthening and rebuilding the disciplines of physiology, pharmacology, and clinical pharmacology will be necessary to provide the capacity to develop and evaluate new biomarkers and bridge across animal and human studies. The emerging techniques of pharmacogenomics and proteomics show great promise for contributing biomarkers to target responders, monitor clinical response, and serve as biomarkers of drug effectiveness. However, much development work and standardization of the biological, statistical, and bioinformatics methods must occur before these techniques can be easily and widely used. Specific, targeted efforts could yield early results.

Predictive Safety Testing Consortium

The Predictive Safety Testing Consortium (PSTC), a collaboration of industry, academia, and government research groups with the goal of developing safety biomarkers for preclinical studies, and the Coalition Against Major Diseases, both launched by the Critical Path Institute, provide valuable examples of the outcomes and lessons learned by different types of consortia working on new drug development tools (Stephenson and Sauer 2014). PSTC submitted its first biomarker "qualification package" for FDA review in 2007, marking the first such submission for the public-private consortium, which now counts several pharmaceutical companies among its participants. In addition to nephrotoxicity, the PSTC also has working groups developing biomarkers for hepatotoxicity, vascular injury, and non-genotoxic carcinogenicity. In 2008, ClinXus (http://www.clinxus.com/), a non-profit biomarker alliance, joined PSTC. ClinXus was formed with the goal of introducing molecular biomarkers into the clinical trial process and uses the expertise and services of each member institution to provide a single point of contact for clinical research clients, as well as patients and physicians that participate in clinical studies.

A panel of biomarkers may ultimately be necessary to account for variations in time course and across different species. So far, the hepatotoxicity group has identified four candidate biomarkers, the carcinogenicity group has selected two mRNA signatures using microarrays that it plans to evaluate further using RT-PCR, and the vascular injury group is still examining genomic data with the goal of identifying candidate biomarkers in collaboration with the Vasculitis Clinical Research Consortium. Biomarker qualification is the top goal of the FDA's Critical Path

Opportunities List, and PSTC activities are important for improving the FDA's understanding of biomarkers. The qualified biomarkers must be shown to reduce time, costs, or adverse events in drug development, as well as than current biomarkers. In addition, they must be qualified fairly narrowly in the mechanistic context in which they are to be applied. The FDA has a particular interest to establish a procedure for qualifying safety biomarkers, which differ from efficacy biomarkers in that they must be applicable across many drugs. Efficacy biomarkers, fall under FDA's Drug-Diagnostic Co-Development Concept Paper.

In 2008, the FDA and the EMEA agreed to accept data for seven kidney toxicity biomarkers as part of the drug approval process. The agencies will now accept the results of tests that measure the levels of seven proteins found in urine – KIM-1, albumin, total protein, β2-microglobulin, cystatin C, clusterin, and trefoil factor-3 – that are indicative of drug-induced damage to kidney cells. The biomarkers were developed under auspices of the PSTC by researchers from Merck and Novartis. The agreement is the first use of a framework allowing submission of a single application to the two agencies. Up to now, both FDA and EMEA have required drug companies to submit the results of two blood tests – blood urea nitrogen and serum creatinine – to evaluate renal toxicity. Now, in addition to those tests, the agencies will accept results from the 7 biomarker-based tests as part of the drug-review process. Drug makers are not required to collect this biomarker data, but if they do, it must be submitted to FDA. The 7 new tests provide important advantages over the BUN and creatinine tests. For example, in experiments using rats, current tests can only detect kidney damage a week after it has begun to occur. The new tests, on the other hand, can detect cellular damage within hours. In addition, BUN and serum creatinine can only indicate that damage has occurred somewhere in the kidneys, but the new tests can pinpoint which parts of the kidney have been affected. PSTC has begun to qualify the biomarkers for use in human studies. In 2010, the Japanese Pharmaceuticals and Medical Devices Agency also accepted PSTC's 7-biomarker panel for use in detecting drug-induced kidney injury, which means that data generated using the panel can be submitted to the agency as part of the drug approval process in Japan.

The Twenty-First Century Cures Act and Biomarkers

The twenty-first Century Cures Act, aimed at promoting the speeding of approval of new drugs and devices was passed in the US House of Representatives in 2015 (http://docs.house.gov/meetings/IF/IF00/20150519/103516/BILLS-1146ih.pdf). The bill would encourage the FDA to rely more on biomarkers and other surrogate measures rather than actual clinical end points in clinical trials for assessing the efficacy of both drugs and devices. The FDA already uses surrogate end points in about half of new drug approvals (Downing et al. 2014). Some biomarkers are

accurate predictors of disease risk and can be useful measures of the efficacy of a new drug (such as LDL cholesterol for statins). But though a drug's effect on a biomarker can make approval quicker and less costly, especially if the comparator is placebo, it may not always predict the drug's capacity to improve patient outcomes. Bevacizumab (Avastin) delayed tumor progression in advanced breast cancer but was shown not to benefit patients. Similarly, rosiglitazone (Avandia) lowered glycated hemoglobin levels in patients with diabetes even as it increased their risk of myocardial infarction. In 2013, patients began to receive a new drug for tuberculosis approved on the basis of a randomized trial relying on a surrogate measure of bacterial counts in the sputum — even though patients given the drug in that trial had a death rate four times that in the comparison group, mostly from tuberculosis. These provisions in the legislation would not immediately change FDA approval standards, but they would give the agency greater discretion, backed by congressional support, to approve drugs on the basis of less rigorous data (Avorn and Kesselheim 2015).

From Validated Biomarker Assay to a Clinical Laboratory Diagnostic

Figure 19.3 shows steps for a validated biomarker assay to a clinical laboratory diagnostic.

Fast Path Programs

The Critical Path Institute was created to support the FDA in its effort to implement the Critical Path Initiative (CPI). It does this by creating innovative programs in education and research to enable the safe acceleration of medical product development. Fast Path programs will focus on applied research and education with respect to the use of biomarkers, clinical trial design, and use of FDA archived data and other aspects of the Critical Path Initiative (CPI) to accelerate drug development. The Institute will create the Efficacy Biomarker Initiative dedicated to fostering the identification, validation and application of biomarkers and surrogate endpoints to facilitate more informative drug development. This initiative will be an active facilitator of the FDA's efforts to validate biomarkers and will foster the use of biomarkers as a methodology for accelerating drug development.

Specific programs being instituted by C-Path are in the areas of cancer, imaging and predictive absorption/ distribution/ metabolism/ elimination (ADME). Through biomarker partnerships, C-Path will help foster innovative strategies for developing therapeutics which target certain orphan diseases using CPI approaches.

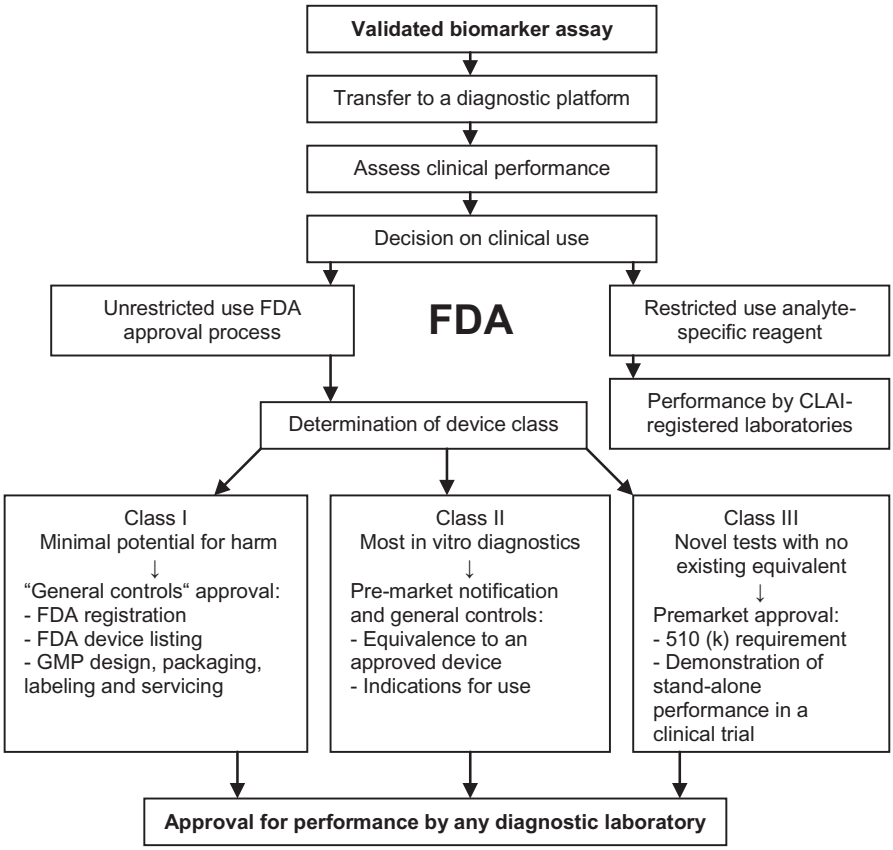

Fig. 19.3 From a validated biomarker assay to a clinical laboratory diagnostic. Abbr: *CLAI* Clinical Laboratory Improvement Amendment, *GMP* Good Manufacturing Practice (Modified from: Kingsmore 2006)

Regulatory Challenges in the Biomarker Field

The current regulatory oversight structure for biomarker tests for molecularly targeted therapies does not include assessment of clinical utility, and no agency or organization in the US is charged with the responsibility of developing evidentiary standards for the clinical utility of biomarker tests (Schott et al. 2015). Given the critical need for evidentiary standards of clinical utility to ensure the implementation of appropriate biomarker tests into routine clinical practice to improve patient care, and the absence of a dedicated body responsible for developing such standards, has called for public-private collaborations to take the lead (Parkinson et al. 2014). Organizations such as the Center for Medical Technology Policy (CMTP) have worked to fill the void by bringing together a range of

public and private stakeholders to work together toward consensus on evidentiary standards for clinical utility. According to a report issued in 2016 by the National Academies of Sciences, Engineering, and Medicine, the regulatory framework for biomarker tests for molecularly targeted therapies presents a number of challenges (Craig 2016):

- There is concern over the adequacy of the current approach to the regulation of biomarker tests for molecularly targeted therapies.
- The processes for regulatory and reimbursement decisions are not currently aligned.
- There is a lack of clearly communicated information about the performance characteristics and intended use of biomarker tests, particularly given the availability of multiple tests for the same purpose.

All these challenges cause uncertainty and confusion among health care providers, patients, test manufacturers, and payers, and in some cases may potentially expose patients to harm.

- To realize the potential of biomarkers in cancer medicine, a single federal agency is needed that would coordinate and oversee a more organized approach to the discovery and development of these biomarkers.
- Diagnostic and pharmaceutical companies and federal agencies should partner to create international research consortia that would generate and share data. These companies would employ resources from different partners that would benefit the entire field.
- The NIH, the NCI, and similar agencies should support cell and tissue repositories that could be used to validate new biomarkers.
- Those who sponsor research should focus on identifying indicators of cell communication pathways that are involved in many kinds of cancer and other diseases so the biomarkers would be broadly applicable.
- Biomarkers for particular drugs are riskier investments because the drugs could fail.
- Underscoring the need for uniform standards is the amount of "false starts" related to biomarker-based breast cancer tests. To remedy that, government agencies and other stakeholders should cooperate to offer guidelines for the development, validation, and use of biomarkers.

FDA Requirements of Biomarkers and Companion Diagnostics

The FDA has recommended companion companion diagnostics for ~60 drugs and requires biomarker/companion diagnostic information in the label of 14 drugs listed in Table 19.2; 9 of these are anticancer drugs.

Table 19.2 Drugs requiring biomarker/companion diagnostic information in the label

Drug	Therapeutic area	Biomarker(s)
Atorvastatin	Metabolic & Endocrinology	LDL receptor
Cetuximab (Erbitux)	Oncology	EGFR, KRAS
Dapsone	Dermatology & Dental	G6PD
Dasatinib	Oncology	Philadelphia chromosome
Imatinib (Gleevec)	Oncology	C-Kit, FIP1L1-PDGFRa fusion, Philadelphia chromosome, PDGFR gene rearrangement
Lapatinib	Oncology	Her2/neu
Maraviroc	CCR5	CCR5
Nilotinib	Oncology	Philadelphia chromosome
Panitumumab	Oncology	EGFR, KRAS
Sodium Phenylacetate & Sodium Benzoate	Gastroenterology	NAGS; CPS; ASS, OTC, ASL; ARG
Sodium Phenylbutyrate	Gastroenterology	NAGS; CPS; ASS, OTC, ASL; ARG
Tamoxifen	Oncology	ER
Tositumomab	Oncology	CD20 antigen
Trastuzumab (Herceptin)	Oncology	Her2/neu

Source: FDA table of pharmacogenomic biomarkers in drug labels, 2012

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Chapter 20
Future of Biomarkers

Introduction

Unfulfilled Needs in Biomarkers

Unfulfilled needs in biomarkers technologies and applications are shown in Fig. 20.1. This figure represents shaded areas as what has been achieved currently and the rest is unfulfilled and has the potential for further development.

Only important areas of application are represented in this graph. For some diseases, needs are enormous and the availability of biomarkers is nonexistent. Need is the greatest (90%) in CNS disorders, where there are few established biomarkers. Cardiovascular disorders have some biomarkers with need rated at 80%. Among therapeutic areas, cancer has the most activity but still the need for further development is rated 70%. Need for efficacy biomarkers of metabolic diseases at 60% is less than in other areas because have the advantage of having validated surrogate endpoints that are approved by the FDA.

Among applications common to all areas, molecular diagnostics is the area of most relevant for clinical applications with unfulfilled needs of 55%. Drug discovery and development is an areas of greatest interest to the pharmaceutical industry where nearly half of the potential achievements have already taken place and the unfulfilled requirements are rated at 50%. Toxicity biomarkers are needed for all therapeutic areas and are not depicted separately.

Challenges Facing the Biomarker Industry

Although biomarkers are promising, a number of challenges have to be met for successful implementation of biomarkers in various programs in drug development and in healthcare. The cost of starting a biomarker discovery program is high and only

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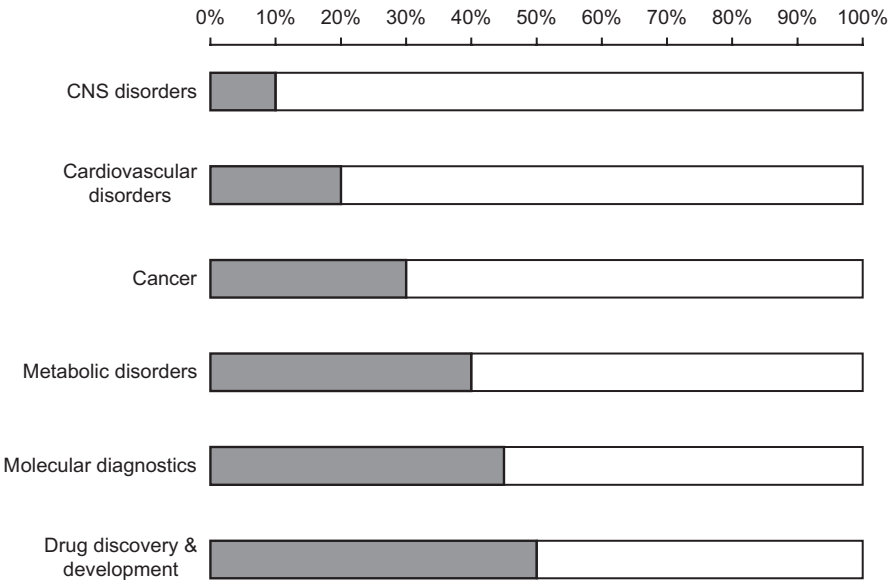

Fig. 20.1 Unfulfilled needs in biomarkers technologies and applications (© Jain PharmaBiotech)

large biopharmaceutical companies have made this initial investment. The returns on this would not be forthcoming until some years later.

Validation of biomarkers is another challenge. However, with the interest of regulatory authorities in this area and increasing cooperation with the industry is facilitating this process. Apart from developing drugs and diagnostics, introduction of these in medical practice is a further challenge.

Pitfalls in the Discovery and Development of Biomarkers

Merely identifying a putative biomarker is very different from showing that it can be validated. The former might require only a matter of weeks and a few patient samples, yet the latter may require multiple multicenter trials. Past failures show how even seemingly straightforward biomarkers can turn out to be false.

A trial for chronic granulomatous disease was originally planned to last only long enough to evaluate whether patients' white blood cells overcame the disease's characteristic defect – an inability to generate a bacteria-killing oxygen burst. Finally a longer trial plan was adopted and showed that the drug had no detectable effect on oxygen production or bacterial death, but still reduced the rate of recurrent serious infections by 70%.

Techniques to discover and interpret biomarkers produce results that vary considerably. For example, mass spectra can be analyzed through machine learning and various computer algorithms to identify sets of peaks that distinguish between, late-stage and early-stage cancer. However, the same biomarker proteins tend to show up in multiple unrelated diseases, making them useless for accurate diagnosis. There is a need for strong data tying biomarker to clinical outcome.

The technology to discover the ideal biomarkers does not exist yet. Humans make hundreds of thousands of proteins and peptides, some of which are a trillion-fold more common than others, and the most informative biomarkers are probably among the least abundant. The techniques necessary to enrich and fractionate low-abundance proteins are immature. Artifacts of sample preparation and processing are frequently indistinguishable from true peptide profiles. Current attempts to validate biomarkers are inefficient and require improvement.

Application of Biomarkers in Medical Practice

Apart from developing molecular diagnostics based on biomarkers, clinical applications require further efforts by the companies to educate the physicians. Some of the measures are:

- Training of the company representatives in biomarkers and interaction with medical profession and company scientific experts.
- Organizing physician education in biomarker-based diagnostics and related therapeutics.
- Scientific and ethical approach to comparing competitor products that are non biomarker-based.
- Since clinical use of biomarkers is related to the development of personalized medicine in the same time frame (next decade), promotion of products should take consideration for this relationship.
- The company should also provide patient education, and information regulatory about regulatory issues as well as reimbursement.

Future Role of Biomarkers in Healthcare

Questions are still being raised as to whether detecting diseases at their earliest stages actually improves health. Based on the information presented in various chapters of this report, it can be concluded that biomarkers will play an important role in future medicine. Biomarkers will have a considerable impact on diagnostics and will facilitate the development of personalized medicine. Role of biomarkers in the management of various diseases have also been discussed. Biomarkers are already playing an important role in management of cancer. Screening approaches have led to dramatic changes in the outcome of cervical cancer.

A promising area in the future application of biomarkers is that in which diseases can be diagnosed in their earliest stages in hopes of effective intervention. The new classes of sensitive imaging technology such as spiral CT can be made more specific through combination with a biomarker in blood, and disease relapse can be detected earlier through entirely new types of protein biomarkers.

One area not discussed so far is the role of biomarkers in preventive medicine and health education. Biomarker profile of a healthy individual may be used to guide individualized health counseling. Susceptibility to certain diseases may require modifications of the general preventive medicine advice. Future studies need to establish the effectiveness of such an approach.

Applications of Biomarkers Beyond Healthcare

There are several potential applications of biomarkers beyond drug development and human healthcare. Proteomic biomarkers have been used in stem cell research. Applications of biomarkers of infections can be extended to detection of biological agents used in bioterrorism and monitoring human exposure to environmental toxins.

Combating Bioterrorism

An integrated rapid, semiportable, prototype point microbial detection/identification system was proposed for clinical specimens, which is also capable of differentiating microbial bioterrorism attacks from threats or hoaxes by defining the pathogen (White et al. 2002). The system utilizes "flash" extraction/analytical system capable of detection/identification of microbes from environmental and clinical matrices. The system couples demonstrated technologies to provide quantitative analysis of lipid biomarkers of microbes including spores in a system with near-single cell (amol/microl) sensitivity. Tandem mass spectrometry increases specificity by providing the molecular structure of neutral lipids, phospholipids, and derivatized spore-specific bacterial biomarker, 2,6-dipicolinic acid as well as the lipopolysaccharide-amide-linked hydroxy-fatty acids of gram-negative bacteria. The extraction should take about an hour for each sample but multiple samples can be processed simultaneously. MALDI-TOF MS specific biomarkers have been shown to be an effective tool for identifying microorganisms. This technique can detect the obligate intracellular bacterium *Coxiella burnetii*, a category B bioterrorism agent.

Biomarkers for Monitoring Human Exposure to Environmental Toxins

Establishing associations between environmental agents and disease presents challenges to both epidemiologists and toxicologists, particularly in cases of complex gene-environment interactions and when there is a long latency between

exposure and disease. The epidemiologic value of a biomarker lies in its ability to predict backward toward exposure and forward toward risk of clinical outcome. In 1995, the World Health Organization recognized that biological markers can potentially improve the way in which exposure to environmental factors is assessed. However, only a few valid biological markers were available at that time, which could be effectively used in epidemiological studies and the assessment of risk.

The need for new approaches to assess DNA damage has been increasing due to the implications that different insults on genetic material may have on human health. In this context, the identification of how chemical agents with different mechanisms of action (i.e., antineoplastic drugs) damage DNA provides a good model to investigate some cellular and molecular mechanisms underlying the basis of genetic toxicology. The nasal epithelium is the first barrier with which environmental pollutants interact, and for this reason this epithelium can be useful as a sentinel in order to assess the interactions between the environment and the living organisms. Taking these phenomena into account and using a simple, sensitive and rapid method such as the single cell gel electrophoresis, we could obtain information and an initial approach on the DNA status. This assay in combination with other techniques that provide more information about other molecular parameters could give us a better view of the biological status of the living cell.

Identification of how chemical agents with different mechanisms of action damage DNA provides a good model to investigate some cellular and molecular mechanisms underlying genetic toxicology. The nasal epithelium is the first barrier with which environmental pollutants interact and can be useful for assessing the interactions between the environment and the living organisms. It is possible to assess the DNA status by use of a simple, sensitive and rapid method such as the single cell gel electrophoresis. This assay in combination with techniques that provide more information about other molecular biomarkers could provide a better view of the biological status of the living cell.

Organophosphorus (OP) compounds are still among the most widely used insecticides, and their main mechanism of acute toxicity is associated with inhibition of acetylcholinesterase. Measurements of urine metabolites and of blood cholinesterase activity are established biomarkers of exposure to OPs and of early biological effects. In recent years, increasing attention has been given to biomarkers of susceptibility to OP toxicity. Polymorphisms of paraoxonase (PON1), a liver and serum enzyme that hydrolyzes a number of OP compounds, plays a role in modulating the toxicity of Ops. It is important to determine PON1 status, which encompasses the PON1192Q/R polymorphism (that affects catalytic ability toward different substrates) and PON1 levels (which are modulated in part by a C-108 T polymorphism) over straight genotyping. Epidemiological studies on OP-exposed workers that include assessment of PON1 status to validate in human populations the role of PON1 as a determinant of susceptibility to OPs, as indicated by animal studies, are needed. Documentation of exposure and of early health effects would be most relevant to increase the predictive value of the test.

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