Chapter 1

# Non-Alcoholic Steatohepatitis, Liver Cirrhosis and Hepatocellular Carcinoma: The Molecular Pathways

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**Abstract**

Non-alcoholic steatohepatitis (NASH) is growing into global problem, mainly due to NASH-induced cirrhosis and hepatocellular carcinoma (HCC), that can develop either subsequently to cirrhosis or preceding it. In addition, NASH-induced cirrhosis constitutes a significant fraction of cases diagnosed as cryptogenic cirrhosis. Thus, there is a need for deeper understanding of the molecular basis, leading to liver steatosis, then—to the associated inflammation seen in NASH, loss of liver architecture and cirrhosis, followed or paralleled by carcinogenesis and HCC. Insulin resistance, increased hepatic iron level, and certain cytokines, including TNF-$\alpha$ and IL-6 derived from extrahepatic adipose tissues, can trigger the chain of events. The imbalance between leptin and adiponectin is important as well. These markers remain important during the whole course from NASH through liver cirrhosis to HCC. The molecular pathogenesis substantiates treatment: hypertriglyceridemia can be lowered by low calorie diet; mTOR complex can become inhibited by physical activity and metformin; cholesterol synthesis, RAF/MAPK1/ERK and p21 pathway by statins; inflammation by pentoxyfillin, and kinases (in HCC) by sorafenib. Bidirectional regulation of telomere attrition, senescence and p21 pathway, restoration of wild-type p53 activity and regulation of miRNA network represent attractive future treatment options. Focusing on relevant molecular pathways allows deeper understanding of NASH pathogenesis, leading to identification of predictive markers and treatment targets.

**Keywords:** non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, liver cirrhosis, cryptogenic cirrhosis, hepatocellular carcinoma

## 1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinical and pathological entity with features that resemble alcohol-induced liver steatosis, but, by the definition, it occurs in patients with little or no history of alcohol consumption. NAFLD is subdivided into non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). It encompasses a histological spectrum that ranges from fat accumulation in hepatocytes without concomitant inflammation or fibrosis (simple hepatic steatosis, NAFL) to hepatic steatosis with a necroinflammatory component (inflammation-induced apoptosis in hepatocytes) that may or may not have associated fibrosis. The latter condition, referred to as non-alcoholic steatohepatitis (NASH), can lead to NASH-induced liver cirrhosis (**Figure 1**). In addition, NASH is now recognised as the main cause of cryptogenic cirrhosis [1], as sequential association has been demonstrated in up to 75% of cryptogenic cirrhosis cases (see also Section 3 for detailed discussion of the relationships between NASH and cryptogenic cirrhosis). Liver cirrhosis may further lead to hepatocellular carcinoma (HCC), the most common primary liver cancer known for its poor clinical outcome and limited therapeutic options. Although previously it was considered that risk of HCC is limited to cirrhotic patients [2], a significant fraction of NASH-associated HCC develops in liver showing none or mild fibrosis. The association between NAFLD/NASH and increased HCC risk is supported by strong epidemiologic evidence.

In the year 2010, the annual incidence of HCC in the population of the USA was at least 6 per 100,000. The mortality rate was almost identical to the incidence underscoring the serious prognosis [3]. Patients with NAFLD/NASH are subjected to an increased lifetime risk of HCC. In a 16-year follow-up study, the standardised incidence ratio of HCC in patients with NAFLD/NASH was 4.4 [4]. In a recent global meta-analysis, the HCC incidence among NAFLD patients reached 0.44 (range, 0.29–0.66) per 1000 person-years [5]. The HCC-related mortality rates among NAFLD patients range from 0.25 to 2.3% over 8.3 and 13.7 years of follow-up, respectively [5, 6]. NAFLD/NASH-associated HCC is believed to be the leading cause of obesity-related cancer deaths in middle-aged men in the USA [4]. Consistently, the proportion of HCC related to NAFLD/NASH is increasing worldwide and is reported to range between 4 and 22% in Western countries [7]. Although the exact burden of HCC associated with NAFLD/NASH still remains uncertain, it seems evident that NAFLD and NASH will become the most common causative/risk factors for HCC, surpassing viral or alcohol-related cirrhosis in the future [7]. In the USA, the number of NAFLD-associated HCC cases is annually growing (2004–2009) for 9% [8], while decreased burden of viral hepatitis-induced HCC might be expected due to the achievements in antiviral treatment targeting hepatitis C virus [9].

NAFLD is the major hepatic manifestation of obesity and associated metabolic conditions. The epidemiology of NAFLD mirrors the recent spread of obesity and diabetes. With increasing prevalence of these conditions, NAFLD has become the most common liver disorder in USA [10] and other Western industrialised countries, facing high occurrence of the major risk factors for NAFLD, namely, central obesity, type 2 diabetes mellitus, dyslipidemia and metabolic syndrome [11]. In a recent meta-analysis of 86 studies, comprising 8,515,431 persons from 22 countries, the global prevalence of NAFLD was 25.24% (95% confidence interval [CI], 22.10–28.65) showing the highest occurrence in the Middle East and South America and the lowest in Africa [5].

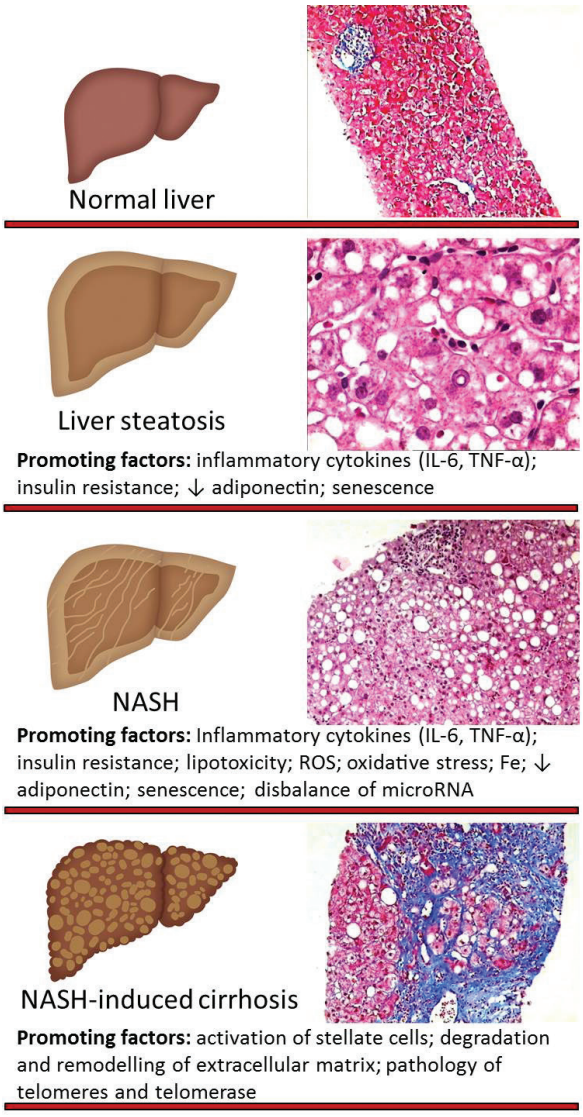


**Figure 1.** Progression of NAFLD. Abbreviations: IL, interleukin; TNF, tumour necrosis factor; ROS, reactive oxygen species; Fe, accumulation of iron compounds.

Thus, 90% of patients suffering from morbid obesity (defined as having body mass index 40 kg/m$^2$ or higher) and 74% patients affected by diabetes mellitus develop NAFLD. In addition, NAFLD has been observed even in non-obese, non-diabetic patients who have increased insulin levels in blood and resistance to insulin action. Consequently, NAFLD affects up to 20–30% of adults in Europe and 46% in the USA: a tremendously high prevalence for a condition that can cause any significant complications [9, 10].

Most patients are diagnosed with NAFLD in their 40s or 50s. Studies vary in regard to the gender distribution of NAFLD, with some suggesting that it is more common in women and others suggesting more frequent occurrence in men [11, 12].

Since 1998, non-alcoholic fatty liver disease has been considered a condition with a "two-hit" course of pathogenesis, first proposed by Day and James [13], describing the role of lipid peroxidation in liver injury. The "first hit" is the development of hepatic steatosis. It was suggested that hepatic triglyceride accumulation increased the susceptibility of the liver to the "second injury hit" by inflammatory cytokines and/or adipokines, mitochondrial dysfunction and elevated oxidative stress that together promote steatohepatitis and fibrosis [14]. Alternatively, many factors may act simultaneously leading to the development of NAFLD: this hypothesis corresponds to the multihit model proposed by Tilg and Moschen [15].

Experimental and population studies have shown the links between NAFLD/NASH and development of HCC. However, the mechanisms by which NASH progresses to HCC are only beginning to be elucidated [14]. NASH is the most rapidly growing risk for liver transplantation because of HCC. Wong et al. in their study included 61,868 patients over the period 2002–2012 and found that the proportion of NASH-related HCC increased from 8.3 to 13.5%, an increase of near 63% [16].

This increase is alarming as HCC already is the fifth most frequently diagnosed cancer and the second leading oncologic death cause worldwide [17], with increasing incidence and mortality rates in Europe [18]. Thus it is crucial to analyse molecular pathways involved in NASH-induced cirrhosis and HCC carcinogenesis. Focusing on the molecular events involved in pathogenetic chain of events from NASH to liver cirrhosis and HCC would provide not only better theoretical understanding of liver diseases preceding and following cirrhosis but would also allow to recognise predictive markers and treatment targets before HCC development.

## 2. Common pathogenetic mechanisms of NAFLD

Hepatic steatosis or excessive triglyceride accumulation in the liver is a prerequisite to the histological diagnosis of NAFLD. Several mechanisms may lead to steatosis, including (1) increased fat supply because of high-fat diet or excess lipolysis in adipose tissues, which increase free fatty acid (FFA) level; (2) decreased fat export in the form of very low density lipoprotein-triglyceride complex, secondary to either reduced synthesis of the relevant proteins or compromised excretion; (3) decreased or impaired β-oxidation of FFA to adenosine triphosphate and (4) increased hepatic synthesis of fatty acids through *de novo* lipogenesis [1, 19]. Free fatty acid delivery to the liver accounts for almost two-thirds of its lipid accumulation. *De novo* lipogenesis therefore only contributes to the accumulation of hepatic fat in case of NAFLD [15].

The molecular mechanisms responsible for the accumulation of fat in the liver are complex (**Figure 2**). Certain inflammatory cytokines, particularly those derived from extrahepatic

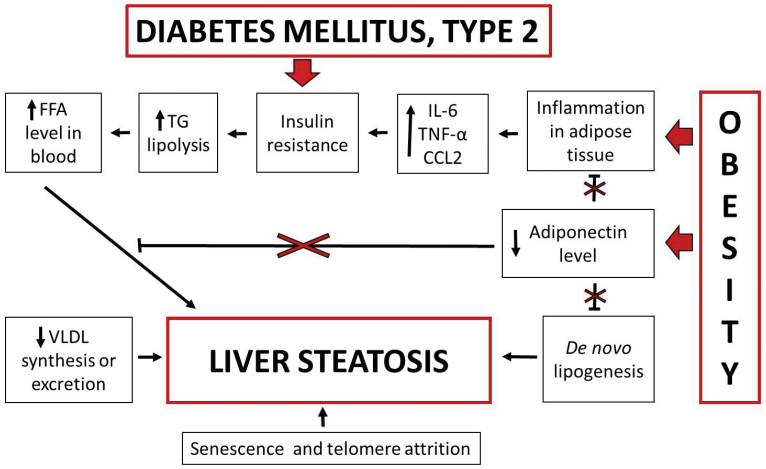


**Figure 2.** Pathogenesis of liver steatosis. Abbreviations: FFA, free fatty acids; TG, triglycerides; IL, interleukin; TNF, tumour necrosis factor; CCL2, CC motif chemokine ligand 2; VLDL, very low density lipoproteins.

adipose tissues, can trigger this process. Insulin resistance appears to be at the centre for the massive metabolic dysregulations that initiate and aggravate hepatic steatosis. At a certain point, the simple steatosis transforms to steatohepatitis in about 20–30% of NAFLD patients [19]. A major feature in the transition from NAFLD to NASH is the appearance of hepatic inflammation [14]. This breakthrough-like process is mediated by the interplay of multiple hit factors and is orchestrated by rich network of miRNAs [20]. Currently, a number of common pathogenetic mechanisms have been proposed and characterised for the transition from simple steatosis to NASH [19]. A summary of these mechanisms is shown in **Figure 3**.

### 2.1. Inflammation in peripheral adipose tissue

Hypoxia and death of rapidly expanding adipocytes are considered important initiating factors of adipose tissue inflammation in obesity [19]. During inflammation, typical cytokines like tumour necrosis factor (TNF)-$\alpha$, interleukin (IL)-6 and CC motif chemokine ligand 2 (CCL2) are secreted by inflammatory cells infiltrating adipose tissue [21]. TNF-$\alpha$ was the first pro-inflammatory cytokine detected in adipose tissue. TNF-$\alpha$ and IL-6 are involved in the regulation of insulin resistance [19]. TNF-$\alpha$ and IL-6 induce insulin resistance in adipocytes, stimulating triglyceride lipolysis and fatty acid release into the circulation. CCL2 recruits macrophages to the adipose tissue, resulting in even higher local cytokine production and perpetuating the inflammatory cycle [19]. In the liver, increased expression of hepatic IL-6 correlates with higher degree of insulin resistance in patients with suspected NAFLD [1].

At the same time, extrahepatic adipocytes are compromised in their natural ability to secrete adiponectin, an anti-inflammatory adipokine that facilitates the normal partitioning of lipid

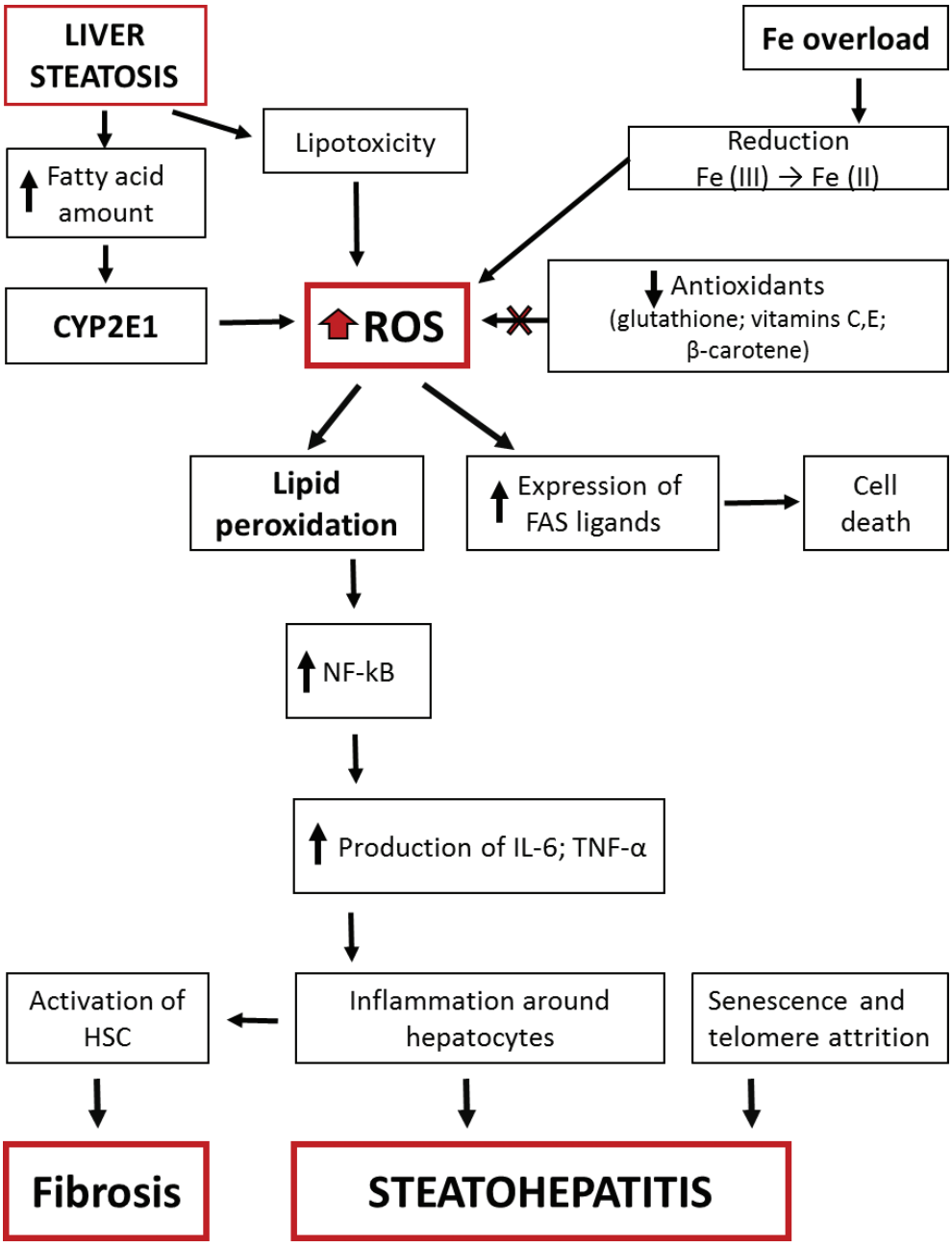


**Figure 3.** Pathogenesis of non-alcoholic steatohepatitis. Abbreviations: CYPE1, cytochrome CYP2E1; ROS, reactive oxygen species; Fe, iron; NF-κB, nuclear factor kappaB; IL, interleukin; TNF, tumour necrosis factor; HSC, hepatic stellate cells.

to adipocytes for storage [19]. Adiponectin is a hormone secreted exclusively by adipose tissue. It has beneficial effects on lipid metabolism. In the liver, adiponectin is considered to have insulin-sensitising, anti-fibrogenic and anti-inflammatory properties by acting on hepatocytes, liver stellate cells and hepatic macrophages (Kupffer cells), respectively. Adiponectin suppresses the transportation of free fatty acids to the liver as well as gluconeogenesis and *de novo* synthesis of fats but enhances oxidisation of FFAs [21]. The adiponectin-induced suppression of aldehyde oxidase and transforming growth factor has net anti-fibrotic effect [21], while decreased release of pro-inflammatory cytokines including TNF-$\alpha$ reduces inflammation [1]. Decreased levels of adiponectin result in loss of these protective metabolic, anti-fibrotic and anti-inflammatory effects.

Together, these abnormalities accentuate fat loss from adipocytes and promote ectopic fat accumulation [19].

### 2.2. Insulin resistance

Obesity and type 2 diabetes mellitus, both conditions associated with peripheral insulin resistance, are frequently diagnosed in patients affected by non-alcoholic fatty liver disease [12]. Evaluating patients suffering from diabetes mellitus, NAFLD was found in 74% of them in North American study, 70% in Italian population and 35–56% in Eastern countries. In Mexico, prevalence of NASH in diabetics was 18.5%. The prevalence of NAFLD in obese patients is 57–90% in Western and 10–80% in Eastern populations. NASH is present in 15–20% patients affected by obesity. The frequency of NASH is higher in those undergoing bariatric surgery and can reach 48–60% in USA men, 20–31% in USA females and up to 80% in Taiwan patients [9, 10, 12].

Insulin resistance has also been observed in NASH patients who are not obese and those who have normal glucose tolerance [1]; however, not all people with NAFLD have increased insulin resistance. NAFLD also cannot be considered as a cause for insulin resistance but rather as a consequence [19].

Resistance to the action of insulin results in important metabolic changes, including the turnover of lipids. It is characterised not only by increased circulating insulin levels but also by increased hepatic gluconeogenesis, impaired glucose uptake by muscle, enhanced peripheral lipolysis, increased triglyceride synthesis and increased hepatic uptake of fatty acids, as well as increased release of inflammatory cytokines from peripheral adipose tissues, which are the key factors promoting accumulation of liver fat and progression of hepatic steatosis [1, 19].

### 2.3. Lipotoxicity

The term "lipotoxicity" describes the deleterious effects of excess FFA and ectopic fat accumulation resulting in organ dysfunction and/or cellular death. In obesity, excessive food intake combined with high FFA output from insulin-resistant adipose tissue surpasses the storage and oxidative capacity of tissues such as skeletal muscle, liver, or pancreatic β-cells [22]. Long-chain saturated fatty acids, as well as free cholesterol derived from *de novo* synthesis can be harmful to hepatocytes. Free cholesterol accumulation leads to liver injury through

the activation of intracellular signalling pathways in Kupffer cells, liver stellate cells, and hepatocytes [19], ultimately promoting inflammation and fibrosis [23]. FFAs are redirected into noxious pathways of nonoxidative metabolism with intracellular accumulation of toxic metabolites. It is not TG accumulation *per se* that is uniquely hazardous, but rather the lipid-derived metabolites that trigger the development of reactive oxygen species (ROS) and activation of inflammatory pathways [22], including up-regulation of nuclear factor kappaB, production of TNF-$\alpha$ and IL-6 [24], and the subsequent inflammatory reaction in the liver [1].

### 2.4. Oxidative stress

In the context of increased supply of fatty acids to hepatocytes, oxidative stress can occur. It is attributable to the raised levels of reactive oxygen/nitrogen species and lipid peroxidation that are generated during free fatty acid metabolism in microsomes, peroxisomes, and mitochondria [19]. NAFLD and NASH-induced oxidative stress is partly regulated through cytochrome P450 2E1 (CYP2E1) as it metabolises C10–C20 fatty acids [14] that in turn produce hepatotoxic free oxygen radical species [1]. Peroxidation of plasma and intracellular membranes may cause direct cell necrosis/apoptosis and development of megamitochondria, while ROS-induced expression of Fas-ligand on hepatocytes may induce fratricidal cell death [19]. Recent studies support the idea that oxidative stress may be a primary cause of liver fat accumulation and subsequent liver injury [25], as well as ROS may play a part in fibrosis development. Lipid peroxidation and free oxygen radical species can also deplete antioxidant stores such as glutathione, vitamin E, beta-carotene, and vitamin C, rendering the liver susceptible to oxidative injury [1].

### 2.5. Increased hepatic iron concentration

The degree of liver fibrosis in nonalcoholic steatohepatitis shows correlation with the concentration of iron compounds in the hepatocytes. The underlying mechanism might involve the ferric-to-ferrous reduction (switch of trivalent Fe(III) to divalent Fe(II) compounds), resulting in simultaneous production of free oxygen radicals [1]. In addition, sinusoidal iron accumulation might also have a pathogenetic role in the progression of chronic liver diseases and development of hepatocellular carcinoma [26]. However, at least in Eastern populations, disturbances of iron metabolism are rarely observed in NAFLD patients [12]. In patients without iron overload, increased ferritin level in the blood may still be associated with insulin resistance and fatty liver [27].

### 2.6. MicroRNAs in NAFLD

MicroRNAs are small molecules of non-coding RNA that act as large-scale molecular switches. The pathogenetic chain of events in the transition to NAFL, NASH, and liver cirrhosis is richly regulated by miRNA network: it has been estimated that approximately 54 miRNAs regulate 107 genes involved in the development of NAFLD. The up-regulation of miR-26b and down-regulation of miR-26a decrease insulin sensitivity, while lower levels of miR-451 are associated with pro-inflammatory background. The up-regulation of miR-155 and miR-107 promotes fat accumulation in liver cells. Enhanced fibrosis is mediated by miR-21. Assessing

patients with NAFLD-associated liver fibrosis, at least 9 miRNAs are expressed in modified levels, including higher expression of miR-31, miR-182, miR-183, miR-224, and miR-150 as well as down-regulated levels of miR-17, miR-378i, miR-219a, and miR-590. In the progression of liver fibrosis, the normally high levels of miR-22 and miR-125b are suppressed. The miR-29 family showing anti-fibrotic action in many organs is also suppressed [20].

## 3. NASH-induced liver cirrhosis

Liver cirrhosis develops (**Table 1**) when simple steatosis progresses to steatohepatitis and then fibrosis [11]. The composition of the hepatic fibrosis is similar regardless of the cause of injury as it follows the paradigm for wound healing in other tissues, including skin, lung and kidney. Fibrosis occurs first in regions of most severe injury over several months to years of ongoing tissue damage [23, 28, 29].

| Targets | Involved cells or molecules | Result |
|---|---|---|
| Stellate cells | Activated stellated cells are transformed to proliferating, fibrogenic and contractile myofibroblasts | Remodelling of the matrix |
| Macromolecules in the extracellular matrix | Collagens: the total collagen content increases 3- to 10-fold including an increase in fibril-forming collagens (i.e., types I, III, and IV) and some non-fibril forming collagens (types IV and VI). | The extracellular matrix switches from the normal low-density basement membrane-like matrix to the interstitial type |
| | Glycoproteins: fibronectin, laminin, SPARC, osteonectin, tenascin, and von Willebrand factor | |
| | Matrix-bound growth factors | |
| | Glycosaminoglycans: perlecan, decorin, aggrecan, lumican, and fibromodulin | |
| | Proteoglycans: shift from heparan sulphate-containing proteoglycans to those containing chondroitin and dermatan sulphates | |
| Degradation of extracellular matrix | Matrix metalloproteinase 2 | Disruption of normal matrix facilitates replacement by desmoplastic matrix |
| | Matrix metalloproteinase 9 | |
| | Membrane-type metalloproteinase 1 and/or 2 | |
| | Stromelysin 1 | |

**Table 1.** The key structures in the development of liver cirrhosis.

Cryptogenic cirrhosis is the end stage of a chronic liver disease in which the underlying aetiology remains unknown after extensive clinical, serological and pathological evaluation [30, 31]. In different studies, 3–30% of liver cirrhosis cases have been attributed to the cryptogenic group [9]. Naturally, occasionally the diagnosis of cryptogenic cirrhosis is issued just due to lack of information despite the definition demanding complete investigation. Studying explanted livers of cirrhotic patients undergoing liver transplantation and having preoperative diagnosis of cryptogenic cirrhosis, specific cause was identified in 28.6% of cases. The relevant diagnoses included autoimmune hepatitis, sarcoidosis, primary biliary cirrhosis, sclerosing cholangitis, congenital hepatic fibrosis and Wilson's disease [32]. Other data/investigational methods can yield significant information as well. For instance, a significant fraction of cases initially diagnosed as cryptogenic liver cirrhosis can be associated with occult hepatitis B infection [33].

Recent evidence suggests that cryptogenic cirrhosis is strongly associated with development of HCC, while in a varying percentage (6.9–50%) of HCC, the underlying aetiology of liver disease cannot be determined. In a retrospective study of 641 HCC patients, cryptogenic cirrhosis was found in 44 (6.9%) cases, characterised also by more frequent occurrence of obesity and diabetes mellitus than in patients having history of chronic viral hepatitis and alcohol abuse. Considering the known association between obesity, diabetes and NASH, it was hypothesised that NASH is the precursor of cryptogenic cirrhosis and hepatocellular carcinoma [34].

At present, there is strong evidence that cryptogenic cirrhosis represents the end state of NASH at least in a fraction of patients. First, the progression of fibrosis in NASH is associated with gradual loss of fat vacuoles. Thus, the specific morphological changes would be burned out when the cirrhosis develops. Second, patients diagnosed with cryptogenic cirrhosis have high prevalence of metabolic changes as type 2 diabetes mellitus, obesity, or history of those disorders. If the history of preceding diabetes mellitus or obesity or liver biopsy revealing NAFLD is considered as the diagnostic criteria, 30–75% of cryptogenic cirrhosis cases can be retrospectively associated with NASH [9]. Third, due to growing awareness of the entity of NASH-induced cirrhosis, direct evidence has been brought by data obtained in explanted livers. Cases that were clinically diagnosed as cryptogenic cirrhosis were reclassified as NAFLD (either cirrhosis or pre-cirrhotic stage) in 78.6% of cases [12, 35, 36].

In comparison with liver cirrhosis due to other aetiologies, NASH-induced cirrhosis is diagnosed in older patients. Higher cardiovascular mortality is observed, in addition to the classic complications of liver cirrhosis attributable to portal hypertension and oesophageal variceal bleeding, infections and renal failure [9].

In a population-based, large study, carried out in the United Kingdom, the following distribution of cirrhosis by the cause was found (in patients, diagnosed in 1987–2006): alcohol-induced, 56.1%; cryptogenic, 20.8%; attributable to viral hepatitis, 12.0%; autoimmune or metabolic (i.e., in this study—haemochromatosis or alpha-1-antitrypsin deficiency), 11.0% [37]. In a nationwide Danish study regarding 11,605 patients diagnosed with liver cirrhosis in 1977–1989, 61.7% of cases were alcohol-induced, 2.8%—attributable to primary biliary cirrhosis, 14.6%—related to chronic hepatitis (including autoimmune inflammation) and 20.9%—

non-specified [38]. Regarding the cause of cirrhosis in explanted livers, 48.6% were related to chronic viral hepatitis (31.1% to HCV and 15.9% to HBV, 1.6% to HCV and HBV coinfection), 23.1% to alcohol-induced liver damage and 16.7% to NAFLD [36]. The data on explanted livers may not reflect the true incidence of NASH-induced cirrhosis as NAFLD patients are less likely to receive transplant. The probability to receive liver transplant within 1 year is 40.5% in NAFLD, contrasting with 47% for hepatitis C or alcohol-induced cirrhosis. The difference is the result of several factors: contraindications due to morbid obesity, comorbidities, older physiologic age, impaired renal function as well as slower disease progression [9].

Thus, cryptogenic cirrhosis is a significant burden for health care systems. Patients undergoing liver transplantation for cryptogenic cirrhosis are subjected to higher postoperative mortality, lower cumulative 5- and 10-year survival and higher rate of chronic rejection [32]. NASH is the most rapidly growing indication for simultaneous liver and kidney transplantation. NASH and cryptogenic cirrhosis in patients having body mass index greater than 30 $kg/m^2$ constituted 6.3% in the years 2002–2003 but 19.2% in the years 2010–2011 [39].

As the liver becomes fibrotic, significant changes occur in the extracellular matrix (ECM) quantitatively and qualitatively. ECM refers to macromolecules that comprise the scaffolding of either normal or fibrotic liver. These include collagens, non-collagen glycoproteins, matrix-bound growth factors, glycosaminoglycans, proteoglycans and matricellular proteins. In case of fibrosis, the total collagen content increases 3- to 10-fold including an increase in fibril-forming collagens (i.e., types I, III and IV) and some non-fibril forming collagens (types IV and VI). Glycoproteins (fibronectin; laminin; secreted protein, acidic and rich in cysteine: SPARC; osteonectin; tenascin, and von Willebrand factor), proteoglycans and glycosaminoglycans (perlecan, decorin, aggrecan, lumican, and fibromodulin) also accumulate in cirrhotic liver. Particularly notable is the shift from heparan sulphate-containing proteoglycans to those containing chondroitin and dermatan sulphates. These processes represent a change in the type of ECM in subendothelial space from the normal low-density basement membrane-like matrix to the interstitial type.

The replacement of the low-density matrix with the interstitial type influences the function of hepatocytes, liver stellate cells, and endothelium of blood vessels: the microvilli disappear on the surface of liver parenchymal cells, and endothelium loses fenestrations precluding effective molecule exchange between blood and liver parenchyma. In addition, stellate cells undergo activation [23].

The hepatic stellate cell is the primary source of ECM in normal and fibrotic liver. Hepatic stellate cells, located in subendothelial space of Disse between hepatocytes and sinusoidal endothelial cells, represent one-third of the non-parenchymal population or approximately 15% of the total number of resident cells in normal liver. Stellate cells comprise a heterogeneous group of cells that are functionally and anatomically similar but differ in their expression of cytoskeletal filaments, retinoid content, and potential for activation. Stellate cells with fibrogenic potential are not confined to liver and have been identified in other organs such as the pancreas, where they contribute to desmoplasia in chronic pancreatitis and carcinoma. Hepatic stellate cell activation is the common pathway leading to hepatic fibrosis. During activation, stellate cells undergo a transition from a quiescent vitamin A-rich cell into

proliferating, fibrogenic, and contractile myofibroblasts [23], which have strong ability to secrete collagen and migrate to the area of necrosis and inflammation [40]. Proliferation of stellate cells occurs predominantly in regions of greatest injury.

Considering liver fibrosis, the balance between synthesis and degradation of extracellular matrix also is of importance as enhanced destruction of the normal matrix in the space between hepatocytes and endothelial cells leads to accumulation of dense scar tissue. Degradation occurs through the actions of at least four enzymes: matrix metalloproteinase (MMP) 2 and MMP9, which degrade type IV collagen; membrane-type metalloproteinase 1 or 2, which activate latent MMP2 and stromelysin 1, which degrades proteoglycans and glycoproteins and activates latent collagenases. Stellate cells are the principal source of MMP2 and stromelysin. Activation of latent MMP2 may require interaction with hepatocytes. Markedly increased expression of MMP2 is a characteristic of cirrhosis. MMP9 is secreted locally by Kupffer cells. Disruption of the normal liver matrix is also a prerequisite for tumour invasion and stromal desmoplasia.

The cytochrome CYP2E1 may have an important role in the generation of reactive oxygen species that stimulate liver stellate cells. Cultured hepatic stellate cells grown in the presence of CYP2E1-expressing cells increase the production of collagen, an effect prevented by antioxidants or a CYP2E1 inhibitor. These data suggest that the CYP2E1-derived reactive oxygen species are responsible for the increased collagen production. Such findings may help to explain the pathogenesis of liver injury in alcoholic liver disease since CYP2E1 is alcohol inducible. As noted above, reactive oxygen species are generated through lipid peroxidation from hepatocytes, macrophages, stellate cells, and inflammatory cells. In alcoholic or non-alcoholic steatohepatitis, ROS generation in hepatocytes results from induction of cytochrome P450 2E1, leading to pericentral (zone 3) injury. Also, oxidase of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) mediates fibrogenic activation of hepatic stellate cells, as well as of Kupffer cells or resident liver macrophages through generation of oxidative stress. Increasing knowledge about NADPH oxidase isoforms and their cell-specific activities is leading to their emergence as a therapeutic target [23].

Pathology of telomeres and the related molecular events represent another key mechanism that is associated both with induction of liver steatosis and progression of NAFLD [41]. Telomerase mutations can accelerate progression of chronic liver disease to cirrhosis [42]. Missense mutations in telomerase reverse transcriptase hTERT are found more frequently in cirrhosis regardless of aetiology [41]. Thus, missense mutations were observed in 7% of cirrhotic patients in USA [43]. Functional mutations were identified in 3% of German patients affected by cirrhosis [44].

Telomeres are repeated, short DNA sequences (in humans—TTAGGG) located at the chromosome end. These structures prevent chromosomal end-to-end fusion as well as protect the coding DNA from progressive loss at mitosis. During each mitosis, the DNA polymerase complex cannot replicate the terminal 5′ end of the lagging strand. Consequently, the chromosomal end is lost. Due to the presence of telomeres, this loss is limited to telomeres. However, the telomeres shorten in each mitosis. Telomere attrition is especially marked in chronic diseases associated with increased cell loss and proliferation. When they become critically short, cellular

ageing *s.* senescence and apoptosis follows. To ensure the unlimited proliferation of cancer, malignant cells maintain telomere length via different mechanisms. The most significant ones include telomerase reverse transcriptase hTERT, its RNA template: telomerase RNA component hTERC, the hTERC-protecting and stabilising dyskerin complex (consisting of four nucleolar proteins) and shelterin complex, including six proteins [41].

NAFLD is characterised by telomere shortening and increased cellular senescence in comparison to healthy controls [45]. The changes in telomeres represent an important mechanism in the transition to liver cirrhosis. However, dual effects are observed. In progressing chronic liver disease, cellular senescence enhances the loss of parenchyma, limiting the replicative potential of hepatocytes. In contrast, in advanced liver damage, the ageing of stellate cells stops the remodelling and thus, the further progression of fibrosis. Still another prognostic aspect can be involved regarding HCC development: senescent stellate cells can promote carcinogenesis by secreting pro-carcinogenic mediators. These changes are described as the senescence-associated secretory program [41]. The extent of fibrosis in NAFLD is associated with p21 protein representing another molecular regulator of cellular senescence [41].

Although shorter telomeres are considered a hallmark of liver cirrhosis regardless of aetiology [41], the telomeres in NAFLD patients are shorter than in those affected by cryptogenic cirrhosis. In NAFLD, telomere length correlates with the level of hTERT mRNA, while hTERT-independent mechanisms already start to operate in cryptogenic cirrhosis [45].

## 4. NASH-induced HCC

Although the association between NAFLD and HCC was first observed more than two decades ago, mostly through NASH-induced cirrhosis [11], the molecular events that link NAFLD and HCC are still incompletely understood. Following the general principles of cancerogenesis, HCC in cirrhotic liver develops by dysplasia—carcinoma pathway: from a dysplastic cirrhotic nodule. The process is slow and can last for several decades [34]. The genetic events that are prerequisite for malignant change develop in the background of increased cellular proliferation. Hypothetically, it is possible that the molecular portrait of HCC in DNA, mRNA, microRNA and protein level is different in accordance to the inciting factor of the underlying liver disease. If this is true, specific molecular targets may exist for the diagnostics, prevention or treatment of NASH-induced HCC or HCC arising in diabetic and/or obese patients [10].

The course of HCC that is associated with cryptogenic cirrhosis differs from HCC developing in other clinical settings [46]. HCC also varies by epigenetic signature in accordance to the cause [47].

The risk of hepatocellular carcinoma differs by the aetiology of cirrhosis. To estimate this, a large population-based study was carried out in the United Kingdom. All patients diagnosed with liver cirrhosis were identified, and the results were compared to national cancer registry identifying those diagnosed with HCC. The 10-year cumulative incidence of HCC was 4% in cirrhosis induced by chronic viral hepatitis, 3.2% in cirrhosis due to autoimmune or metabolic (in this study—haemochromatosis, alpha-1-antitrypsin deficiency) diseases, 1.2%

in alcohol-induced cirrhosis and 1.1% in cryptogenic cirrhosis, while the same estimates at 1 year were 1.0, 0.8, 0.3 and 0.3%, respectively. This study has the significant benefit of exploring HCC risk in patients that differ by aetiology of cirrhosis but belong to the same population [37]. Considering patients referred for liver transplantation, the frequency of hepatocellular carcinoma in cryptogenic cirrhosis is lower (8%) than in cirrhosis related to chronic hepatitis B (29%) or C (19%) as reported by Alamo et al. [32]. For the epidemiological estimates of HCC in different liver pathology, see also **Table 2** [37, 38].

The causal distribution of HCC shows geographic variations. Thus, in Canadian patients, 45% of cases were attributable to alcohol-induced cirrhosis, 26% to cryptogenic cirrhosis and 13% to hepatitis C. In patients from Saudi Arabia, 47% of HCC were caused by hepatitis C, 27% by cryptogenic cirrhosis and 21% to hepatitis B [48]. In USA, regarding the HCC cause, 54.9% of cases were induced by HCV, 16.4% by alcohol, 14.1% by NAFLD and 9.5% by HBV [10]. In explanted livers, 81.8% of HCC were associated with viral hepatitis, 9.1% with alcohol-induced liver damage and 9.1% with NAFLD [36].

In the USA, the number of NAFLD-associated HCC cases is annually growing for 9%, if the time span 2004–2009 is evaluated [10]. In Europe, NAFLD-related HCC comprised 35% of all HCC cases in 2010. HCC that is not related to hepatitis B or C is becoming increasingly frequent in Japan as well; however, here, it comprises only 10% of all HCC cases [53]. NASH is responsible for higher percentage of HCC in Western than in Eastern societies [12].

Hepatocellular carcinoma in patients affected by metabolic syndrome has distinct morphology [49]. NAFLD-associated HCC is characterised by larger size [34] and moderate or high differentiation degree [34], showing high differentiation as frequently as in 65% of cases [49]. However, the tumours lack capsule thus confirming the true malignant biological potential [34]. This is an important diagnostic trait considering the association between NAFLD, low-grade HCC [49], and liver adenomatosis [50].

The prognostic estimates are somewhat controversial. The NAFLD-associated hepatocellular carcinomas are diagnosed as more advanced tumours in older patients showing higher cardiovascular morbidity. The patients are less likely to receive liver transplant and have higher

| Estimate | Alcohol-induced cirrhosis | Autoimmune and genetic diseases | Chronic hepatitis | Cryptogenic cirrhosis | Reference |
|---|---|---|---|---|---|
| SIR; 95% CI | 70.6; 59.5–83.2 | 47.0;[1] 12.6–120.2 | 42.7;[2] 25.2–67.3 | 43.4; 30.3–60.4 | Sorensen et al. [38] |
| Incidence rate per 1000 person years; 95% CI | 3.2; 2.1–4.8 | 5.3; 2.6–10.5 | 7.6;[3] 4.3–13.4 | 3.1; 1.6–5.9 | West et al. [37] |

Abbreviations: SIR, standardised incidence ratio; CI, confidence interval.

[1]Primary biliary cirrhosis.

[2]Including viral and autoimmune causes.

[3]Viral hepatitis.

**Table 2.** Epidemiological estimates of hepatocellular carcinoma by the cause of chronic advanced liver pathology.

tumour-specific mortality [10]. HCC associated with cryptogenic cirrhosis is larger than cancers related to HCV even in patients who correspond to Milan criteria [51]. However, after curative treatment, the recurrence risk and mortality are lower for HCC arising in cryptogenic cirrhosis—finding that is in accordance with the grade difference [52].

Although previously it was considered that HCC risk is limited to cirrhotic patients, currently at least 25–30% of NAFLD-related hepatocellular carcinomas develop in the absence of cirrhosis [9]. In Japanese group, 33% of NAFLD-related HCC occurred in the background of none or mild fibrosis contrasting with only 16% in alcohol-induced HCC [53]. According to other researchers, up to 65% of NAFLD-associated HCC evolve in the absence of fibrosis [49]. The proportion of NAFLD-associated HCC developing in non-cirrhotic liver has been variably estimated as 15, 38, or 49% [54–57]. These tumours tend to be larger [57].

The development of HCC in noncirrhotic liver has been associated with malignant transformation in liver cell adenoma [34, 49]. Malignant change in hepatic adenoma correlates with metabolic syndrome [58]. Inflammatory molecular type of liver cell adenoma shows clinical correlation with obesity. The underlying molecular basis could include either activated IL-6 signalling or hyperoestrogenemia associated with obesity. However, a controversy exists here as inflammatory type of liver adenoma is not prone to malignisation [50].

Several pathogenetic ways account for a tumour-promoting environment in obesity and diabetes, allowing to distinguish the pathogenesis of HCC linked to NAFLD from that of viral and other aetiologies.

Obesity has been linked to higher frequency of cancers in a variety of tissues [59, 60] including the liver (**Table 3**). HCC is increasingly diagnosed among obese individuals. In a prospective cohort of the Cancer Prevention Study with more than 900,000 North American subjects, the relative risk of dying from liver cancer among men with a body mass index reaching or exceeding 35 kg/m$^2$ was remarkably higher (4.5 fold) compared to a reference group with normal body weight. In a large cohort involving 362,552 Swedish men, the relative risk of HCC in individuals with a body mass index reaching or exceeding 30 kg/m$^2$ was 3.1 fold higher than in controls having normal weight. Studies from other parts of the world indicate that the link between obesity and increased incidence of HCC has been globally recognised [61].

Obesity has a significant tumour-promoting effect regarding HCC. This effect largely depends on the chronic general low-grade inflammatory response it induces, which involves production of TNF-$\alpha$ and IL-6. Both these molecular mediators are tumour-promoting cytokines [62] and major drivers of cell proliferation in NAFLD and NASH [21]. TNF-$\alpha$ and other mediators produced by activated inflammatory macrophages stimulate compensatory hepatocyte proliferation and expand HCC progenitors. TNF-$\alpha$ further reinforces the inflammatory microenvironment and induces expression of chemokines (CCL2, CCL7 and CXCL13) and growth factors/cytokines (IL-1$\beta$, IL-6, TNF–$\alpha$ itself and hepatocyte growth factor) both by progenitors of hepatocellular carcinoma and surrounding cells [63]. TNF-$\alpha$ up-regulates the cellular proliferation through the molecular pathways of nuclear factor kappaB, mTOR and wide spectrum of kinases. The proliferative and anti-apoptotic activities of IL-6 are largely mediated through the signal transducer and activator of transcription 3, STAT3 [10]. IL-6 also

| Location | Level of evidence |
|---|---|
| Oesophageal adenocarcinoma | Strong |
| Colorectal cancer in males | Strong |
| Pancreatic cancer | Strong |
| Breast cancer | Strong |
| Endometrial cancer | Strong |
| Renal cancer | Strong |
| Multiple myeloma | Strong |
| Liver cancer | Highly suggestive |
| Colonic cancer in females | Suggestive |
| Ovarian cancer | Suggestive |
| Prostate cancer | Suggestive |
| Thyroid cancer | Suggestive |
| Melanoma in males | Weak |

**Table 3.** Obesity-related human cancers [60].

contributes to the metabolic background of cancer sustaining insulin resistance that can be improved by systemic neutralization of IL-6 [64].

Another mechanism involved in the progression of NAFLD to HCC in obese individuals is the imbalance between leptin and adiponectin. Particularly, obesity is linked to increased levels of leptin [34]. Apart from its role in obesity-associated insulin resistance and inflammation, leptin is a pro-inflammatory, pro-angiogenic, and pro-fibrogenic cytokine with a growth-promoting effect by activating the Janus kinase/STAT, phosphoinositide 3-kinase (PI3K)/Akt, and extracellular signal-regulated kinase (ERK) signalling pathways [61]. The up-regulation of PI3K/Akt pathway leads to activation of downstream molecular mediator mTOR that is found in 40% of HCC cases. Leptin-induced up-regulation of mTOR also inhibits autophagy—a process that normally would limit oxidative stress by removing damaged mitochondria. Suppression of autophagy, in turn, increases oxidative tissue damage and subsequent inflammation [21]. Since leptin exerts pro-inflammatory and pro-fibrogenic effects by activating Kupffer cells and stellate cells, it has been associated to disease progression in fibrotic NAFLD [10]. Leptin can also promote invasion and migration of hepatocellular carcinoma cells [65].

Adiponectin, another major adipokine with potent anti-inflammatory, antiangiogenic and tumour growth-limiting properties, is suppressed in obesity [15, 24]. Adiponectin activates 5′-adenosine monophosphate–activated protein kinase, which can suppress tumour growth and increase apoptosis by regulating the mTOR and c-Jun N-terminal kinase/caspase 3 pathways. Moreover, adiponectin opposes the effects of leptin by inhibiting activation of Akt and STAT3, as well as by increasing the expression of SOCS3: the suppressor of cytokine signalling 3 [61].

Thus, low adiponectin levels may be insufficient to suppress endotoxin-mediated inflammatory signalling in Kupffer cells and other macrophages, as well as control angiogenesis, a pivotal mechanism of tumour growth [10]. Microarray analysis of tissue adiponectin levels in HCC patients revealed that adiponectin expression was inversely correlated with tumour size, supporting the hypothesis that adiponectin may inhibit proliferation and dedifferentiation [66].

HCC can show marked accumulation of fat within the neoplastic cells (**Figure 4**). In a study by Salomao et al., 36% of patients who developed HCC in the setting of steatohepatitis were diagnosed as having a steatohepatitic variant of HCC as compared to 1.3% of HCC patients without steatohepatitis [67]. Increased intensity of fatty acid synthesis and characteristic pattern of perilipin proteins has been demonstrated in HCC. Regarding gene expression pattern, activated lipogenesis is associated with higher cell proliferation and worse prognosis in HCC [10]. Hypothetically, HCC cells might benefit from the energetic value of fat compounds or use lipids as building blocks of new cells.

Lipotoxicity, defined as the cellular dysfunction caused by ectopic deposition of fat in non-adipose tissues, may contribute to the development of HCC in NAFLD. Activated oxidation of fatty acids generates high burden of free radicals and lipid peroxide compounds that oxidise and damage large molecules and cell organoids, e.g., mitochondria and endoplasmic reticulum. The damaged cells are subjected to apoptosis, leading to higher activity in liver destruction and progression towards cirrhosis that in turn is closely associated with enhanced proliferation and accumulation of genetic damage. Accumulation of fatty acids may interfere with cellular signalling and promote oncogenesis through altered regulation of gene transcription [10]. Oxidative stress can induce mutations in the tumour suppressor gene *TP53* in a pattern observed in HCC [68].

Adipose tissue expansion, release of pro-inflammatory cytokines, and lipotoxicity collectively promote systemic and hepatic insulin resistance, resulting in hyperinsulinemia [34]. The risk of HCC in patients affected by diabetes mellitus is 2.31 [57]. Insulin resistance and hyperinsulinemia are the most common metabolic features of NAFLD, which correlate with impaired hepatic clearance of insulin and have been linked to tumour development [69]. Deregulated metabolic effects of insulin result in excessive activation of proliferative signalling cascades.

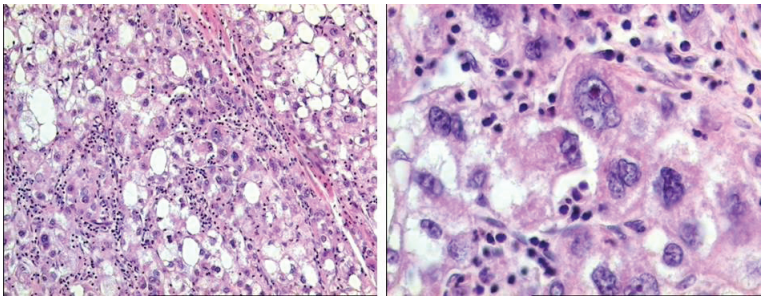

**Figure 4.** Hepatocellular carcinoma showing nuclear atypia and presence of fat in tumour cells. Haematoxylin-eosin stain, original magnification 100× and 400×.

Hyperinsulinemia causes reduced hepatic synthesis of insulin-like growth factor (IGF)-binding protein-1 and increased bioavailability of IGF1, which further promotes cellular proliferation and inhibits apoptosis [10, 34]. It has been shown recently that elevated fasting insulin, which is inversely related to insulin sensitivity, is an independent risk factor for HCC. Baseline serum levels of C-peptide have also been found to be associated with a higher risk of HCC in the general population independently of obesity and other established liver cancer risk factors [69]. Loss of heterozygosity for IGF2 has been observed in over 60% of HCC cases. This likely coincides with IGF2 overexpression, found in HCC, which has been associated to reduced apoptosis and increased cellular proliferation [68].

The importance of insulin resistance is illustrated by the observations that obesity and type 2 diabetes mellitus comprise increased HCC risk even regardless of the presence or cause of liver cirrhosis [9].

A number of studies have demonstrated a critical role for phosphatase and tensin homolog (PTEN) in the progression of NASH to tumour. *PTEN* deletion results in PKB/Akt activation, promoting proliferation and reducing apoptosis. Insulin-like growth factor 2 mRNA binding protein p62 was reported to be a possible upstream regulator of PTEN. Aberrant microRNAs contribute to carcinogenesis. MiR-21 was found to be another upstream regulator of PTEN participating in NASH-associated cancer induction [10, 14, 70].

The oral iron test has revealed increased absorption of iron compounds in patients affected by NASH [71]. In turn, increased amount of iron in liver tissues is associated with increased risk of HCC in patients affected by NASH-related liver cirrhosis [72]. As the reductive conversion of Fe(III) to Fe(II) necessitates increased oxidation of other compounds, oxidative DNA damage can develop and lead to the malignancy [34, 73]. Iron overload also is known to enhance insulin resistance [74] and to act in concert with other factors damaging liver. The significance of iron overload in hepatic carcinogenesis is shown in several models. The risk of HCC is increased in hereditary haemochromatosis, characterised by excessive iron accumulation in the body and caused by excessive absorption because of homozygous C282Y mutation in *HFE* gene. Almost 8–10% of patients with hereditary haemochromatosis develop HCC. Increased relative risk of HCC (10×) has also been demonstrated in association with long-lasting excess dietary iron intake [37, 74, 75]. Thus, there is significant evidence of the carcinogenic action of iron overload, and evidence of iron accumulation in NAFLD and especially NASH that allows drawing conclusion that iron metabolites are contributing to the development of NASH-related HCC.

The expression profile of *Wnt* signalling genes in NASH strongly suggests inhibition of Wnt pathway. IHC staining of β-catenin shows predominately membrane staining with loss of nuclear staining indicating that β-catenin is not active in NASH. In contrast, 20–90% of HCC cases exhibit active Wnt pathway [76]. Thus, the long-lasting conversion of NASH into HCC hypothetically involves up-regulation of Wnt pathway either by activators or loss of inhibitors [77].

Hepatocyte apoptosis is a prominent feature of NASH (**Figure 5**). The executing mechanism of apoptosis includes activation of characteristic lytic enzymes—the caspases. In an apoptotic

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hepatocyte, activated caspase-3 is splitting various cell structures, including cytokeratin (CK) 18—the intermediary filament that represents the specific cytoskeleton protein of hepatocytes. Consequently, blood tests can reveal increased concentration of CK18 fragments [70]. In liver tissues, CK8 and CK18-containing Mallory bodies are evident by light microscopy as large, brightly eosinophilic inclusions in liver cell cytoplasm. Although Mallory hyaline is the hallmark of alcohol-induced hepatitis, its development can also be induced by diet rich in saturated fatty acids. The molecular pathways associated with Mallory body development include IL-6, protein p62 that binds ubiquitin in cell cytoplasm, and reduced concentration of HSP72 that prevents protein misfolding. The presence of CK18 in Mallory bodies correlates with plasma CK18 levels [78]. In a longitudinal paired liver biopsy study, the change of CK18 correlated with disease progression. Patients with increased NAFLD activity score 3 years after initial evaluation had greater increase of plasma CK18 compared with those who had stable or decreased activity score [79]. El-Zefzafy et al. proved that CK18 was a sensitive indicator of the severity of liver disease and also could predict the development of HCC. In their study, the sensitivity and specificity of serum CK18 were 95 and 96.7%, respectively, with a cut-off value of 534.5 U/L for HCC diagnosis [80].

In a study by Salomao et al., devoted to HCC in NASH, immunohistochemically there was diffuse loss of cytoplasmic CK8/18 and an increased number of activated hepatic stellate cells within the steatohepatitic HCC, identical to the pattern seen in the surrounding non-neoplastic liver [67, 81].

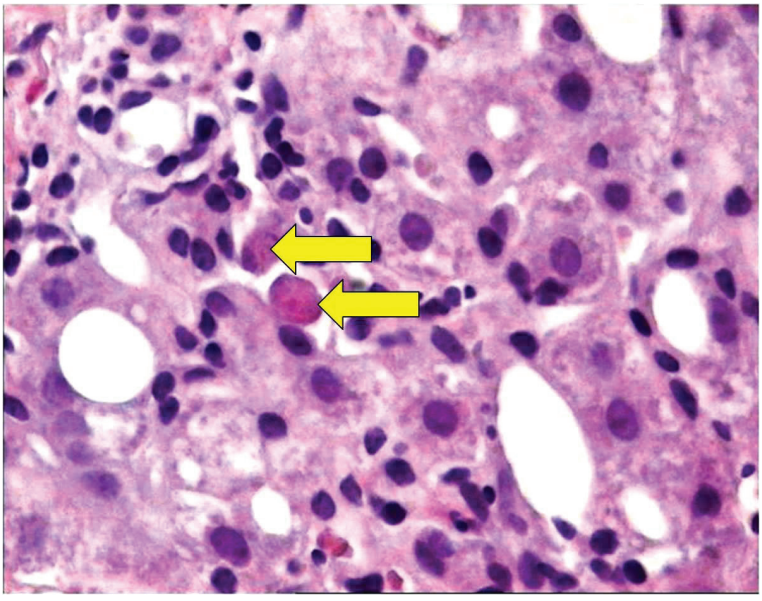

**Figure 5.** Apoptotic bodies (arrows) in non-alcoholic steatohepatitis. Haematoxylin-eosin stain, original magnification 400×.

The HCC development shows complex associations with telomere shortening. The senescence-associated secretory program of liver stellate cells promotes carcinogenesis. The telomere shortening induces also genomic instability thus facilitating HCC development [41]. Indeed, HCC is characterised by significantly shorter telomeres in comparison to adjacent tissues [82]. However, cancer cells still maintain unlimited proliferation. Evidently, hepatocellular carcinoma cells develop compensatory mechanisms either for telomere extension or for cellular proliferation despite telomere shortening. The elongation of telomeres again can be ensured via diverse mechanisms, including hTERT or alternative lengthening of telomeres via telomerase-independent mechanism seen in 7% of HCC cases [41].

Over the progression of HCC, the telomere length changes in contrary direction. Early liver carcinogenesis is associated with telomere shortening, while disease progression is associated with telomere extension, cell immortalisation and reactivation of telomerase [83]. Longer telomeres in HCC are associated with higher stage (regional or distant spread *versus* localised tumour) and grade (III–IV *versus* lower grade) as well as with worse survival [83, 84]. Telomerase promotes HCC development via several pathways, not limited to maintenance of telomeres and thus cellular proliferation. In addition, hTERT can act as a transcription factor in the Wnt molecular cascade [41]. Experimental data by HCC induction in telomerase-deficient mice have shown increased number of early tumours and reduced incidence of high-grade HCC [85].

Interestingly, shorter telomeres are observed more frequently (telomere length ratio between HCC and surrounding tissues lower than the mean, 70.1% *versus* higher, 29.9%) in HCC that is not related to hepatitis B (50.0% *versus* 50.0%) or C (60.0% *versus* 40.0%), or alcohol abuse (50.0% *versus* 50.0%), although the difference does not reach statistical significance [83]. Telomere shortening can be detected in peripheral blood. Notably, this assay can be used to predict HCC persistence (by telomere shortening) in cases attributable to viral hepatitis B or C but not in HCC attributable to non-infectious causes despite comparable size of patient groups [86].

Genetic predisposition has been studied in NAFLD trying to identify those patients that are at particularly increased risk of HCC. The possible candidate genes could be associated with telomere length and mechanisms involved in preserving telomeres [42]. About 10% of patients affected both by HCC and NASH have germline mutations in *hTERT* in comparison to complete absence of such mutations in NASH patients having cirrhosis and healthy controls [41]. In addition, *PNPLA3* polymorphisms have been studied in NAFLD patients, finding twice increased risk of HCC in association with rs738409 C>G. The proposed mechanism involves retinol metabolism in hepatic stellate cells [34].

The interaction of these pathogenetic mechanisms and genetic predisposition finally results in the increased incidence of HCC in NAFLD that reaches 76–201 per 100,000 contrasting with the incidence of 4.9–16 per 100,000 of the general population [57].

## 5. Potential treatment strategies

As no specific treatment is approved for NAFLD, lifestyle interventions play the leading role in NAFLD management. Weight loss due to low calorie diet in combination with physical activities is the main therapeutic approach in overweight patients with NAFLD. As hypertriglyceridemia is a frequent and promoting feature of NAFLD [87] reduction of the triglyceride

level must be among therapeutic goals. In severe hypertriglyceridemia, total fat consumption should be limited to less than 30 g/day, and carbohydrate amount in daily nutrition should be strictly controlled as well [88].

Physical activity has beneficial effect of reducing triglyceride level, even independently from diet [89]. Thus, at least 30 min of moderate activity most days of the week would be a necessary part of dyslipidemia management [90]. Loss of 5% of body weight decreases hepatic steatosis, but body weight loss of 10% could even improve inflammation and fibrosis in liver [87].

Experimentally investigating hepatocyte-specific PTEN-deficient mouse model, Piguet et al. showed that physical activity could reduce HCC growth in fatty liver. In PTEN-deficient mice, HCC incidence was 71% of exercised mice and 100% of sedentary mice. In addition, liver tumour volume in exercised mice was significantly smaller than that of sedentary mice (444 ± 551 *versus* 945 ± 1007 $mm^3$) [91]. The physiological substantiation relies on fact that regular physical activity could inhibit mTOR complex, which is engaged in cell growth and proliferation [92].

Increased hepatic free cholesterol accumulation is typical for NASH. Statins are commonly prescribed to reduce cholesterol synthesis in the liver and thus serum levels of free cholesterol [14]. In a recent European multi-centre cohort study, statin use was associated with protection from steatosis (odds ratio, OR 0.09; 95% CI, 0.01–0.32; p = 0.004), steatohepatitis (OR, 0.25; 95% CI, 0.13–0.47; p <0.001), and fibrosis stage F2–F4 (OR, 0.42; 95% CI, 0.20–0.80; p = 0.017). The protective effect of statins on steatohepatitis was stronger in subjects not carrying the I148M PNPLA3 risk variant (p = 0.02), indicating the role of genetic predisposition [93]. Statins also have been associated with reduced risk (range, 0.46–0.79) of HCC [94].

In a meta-analysis, including 4298 patients with HCC, statin use was associated with a 37% reduction in the risk of hepatocellular carcinoma. The effect was stronger in Asian patients but was also present in Western populations. Moreover, the reduction of cancer risk was independent of statin lipid-lowering effects [95]. Several hypotheses have been proposed, including statin ability to inhibit cell proliferation via inhibition of v-myc avian myelocytomatosis viral oncogene homolog protein phosphorylation which seems to play a role in liver carcinogenesis [96], as well as capacity to inhibit the 3-hydroxy-3-methylglutaryl coenzyme A reductase, which activates multiple proliferative pathways [95]. Simvastatin selectively induces apoptosis in cancer, but not in healthy cells. This proapoptotic effect is maintained via RAF/MAPK1/ERK and growth-inhibitory action by suppression of angiogenesis and proteasome pathway [95, 96]. However, data about liver carcinogenesis and statin effects remain controversial. In another large meta-analysis, including 86,936 participants, no beneficial effect of statin in terms of incidence or death from cancer was observed. Even more, in 67,258 patients who received statins, 35 new liver cancers and 24 deaths from liver cancer were reported showing no significant difference from control group, comprising 67,279 patients who received placebo, and developed 33 new liver cancer (p = 0.93) cases leading to 24 deaths (p = 1.00) as analysed by Carrat [97].

Metformin, a widely prescribed drug for treating type 2 diabetes mellitus, is one of the most extensively recognised metabolic modulators which decreases aminotransferase levels and hepatic insulin resistance. It has no beneficial effects on NAFLD histology but still retains an

important anti-cancer action [87, 98]. The hypothetic antitumor mechanisms of metformin are believed to be (1) inhibition of mTOR, (2) weight loss and (3) suppressed production of ROS and the associated DNA damage, in combination with (4) reduction of hyperinsulinemia, which is known to lead to cell proliferation [99]. In meta-analysis comprising 105,495 patients with type 2 diabetes, Zhang et al. showed that metformin was associated with an estimated 70% reduction in the risk of developing HCC [98]. The risk reduction in metformin users is significant, regarding both incidence (78%) and mortality (77%) from HCC [100].

The mammalian target of rapamycin (mTOR) promotes growth in a majority of liver cancers, including hepatocellular carcinoma. It participates in the formation of two protein complexes—mTORC1 and mTORC2. mTORC1 is sensitive to rapamycin and has ability to activate downstream targets which regulate cellular growth and metabolism. Prolonged mTORC1 activation is related to liver steatosis and insulin resistance in obese patients [14, 101]. Due to the ability suppress mTORC1, rapamycin and its analogues Everolimus and Temsirolimus have been tested to treat HCC. Unfortunately, results have not been promising. In a phase 3 study of patients with advanced HCC, Everolimus increased the frequency of hepatic injury and showed no improvements regarding survival [14]. After 2 weeks with rapamycin treatment, the lipid droplets in the liver decreased, as well as ROS burden. However, rapamycin treatment promoted liver damage with augmented IL-6 and decreased anti-inflammatory IL-10 production, leading to increased hepatic inflammation and hepatocyte necrosis [101].

Inflammation promotes development of complications in patients with cirrhosis contributing to mortality and to liver insufficiency mediated by pro-inflammatory cytokines. The most recognisable pro-inflammatory cytokine associated with liver damage in case of NAFLD is TNF-$\alpha$ that can be inhibited by pentoxifylline. Lebrec et al. performed randomised, placebo controlled, double-blind trial assessing pentoxifyline effect in 335 patients with cirrhosis. Although pentoxifylline had no effect on short-term mortality, it significantly ($p = 0.04$) prolonged the complication-free time span [102].

Knowing the important role of NADPH oxidases (NOXs) and production of ROS in liver fibrosis, different strategies to prevent the oxidative damage have been developed [23]. In hepatocytes, NOX4 mediates suppressor effects on TGF-$\beta$ and can inhibit hepatocyte growth and liver carcinogenesis. In turn, dual NOX4/NOX1 pharmacological inhibitor GKT137831 could decrease both the apparition of fibrogenic markers as well as hepatocyte apoptosis *in vivo* [103].

Currently, multikinase inhibitor sorafenib is the only pharmacological agent that prolongs survival of HCC patients, although the median survival is improved only by 12 weeks [14]. It acts against Raf-1 and B-raf, vascular endothelial growth factor (VEGF) receptors and platelet-derived growth factor receptor kinases [104]. Sorafenib as well as VEGF inhibitors have radiosensitizing effect. However, combined regimens including sorafenib and liver stereotactic radiation or whole liver radiotherapy are characterized by poor tolerability [104]. Various beneficial effects of sorafenib have been reported in liver cirrhosis. As epithelial-mesenchymal transition and TGF-$\beta$ play crucial roles in liver fibrosis, Ma et al. proved that sorafenib had ability to strikingly suppress TGF-$\beta$1 induced epithelial-mesenchymal transition, as well as apoptosis in hepatic stellate cells, in dose-dependent manner [105].

Several treatment strategies might involve the telomere and telomerase complex. In cancer, telomerase inhibitors might arrest tumour growth, prevent further malignisation in surrounding cirrhotic nodules and/or enhance HCC chemosensitivity. In early liver disease, telomerase activation might prevent tissue loss if the etiologic factor cannot be removed. This could be reached via transplantation of liver cells engineered for hTERT expression, direct supply of hTERT to the patient's cells or by small molecules enhancing telomerase activity. However, side effects and enhanced cancer risk must be considered and prevented [41]. The treatment modulating cellular senescence and proliferation control may also target p21 [106–108] and p53 [109] pathways.

The p21 protein, a strong and universal inhibitor of cyclin-dependant kinases, is an important regulator of cell proliferation, apoptosis and senescence [107, 108]. Based on its intracellular location and the molecular background, it can have dual activity. Intranuclear p21 acts as tumour suppressor, as it binds cyclin-dependant kinases and thus suppresses cellular proliferation. Cytoplasmic p21 prevents apoptosis by binding caspases and promotes proliferation and migration of p53-deficient cells. The p21 pathway is also closely associated with senescence. Few small molecular inhibitors of p21 are known, including LLW10, butyrolactone and UC2288. In addition, sorafenib also exhibits anti-p21 activity. LLW10 binds to p21 and induces proteosomal degradation via ubiquitination. Despite the reliable mechanism, the high concentration that is necessary for sufficient activity as well as the instability of LLW10 prevents it from being clinically useful drug. Butyrolactone also induces proteosomal degradation of p21. UC2288 decreases p21 concentration via suppressed transcription and modified posttranscriptional modulation [107]. In turn, upregulation of p21 can be achieved via statins or by anticancer agents including histone deacetylase inhibitors [106]. Induction of senescence would be desirable if the tumour is already present while suppressed senescence might prevent or slow down the development of liver cirrhosis. As was noted, it is possible to modulate p21 level in both directions. However, the net effects must be carefully considered and studied experimentally, knowing the bidirectional activity of p21.

p21 is also an effector of p53-mediated responses in cells maintaining functional p53. In p53-deficient cell, it manifests carcinogenic effects. Thus, restoration of wild-type p53 could be attractive, either in combination with p21-targeted treatment or with other oncological approach. In liver cancer, restoration of p53 activity has resulted in senescence and increased immune response. The therapeutic approaches could include (1) restoration of wild type function to mutant p53 by low molecular weight compounds PRIMA 1 or PRIMA-1MET. The last one has progressed to phase II clinical trials; (2) stabilising p53 due to blocked interaction with MDM2 or MDM4 by nutlins, representing low molecular weight molecules, or by stapled peptides; (3) gene therapy using viral vectors that has already been tested in HCC; (4) induction of synthetic lethality [109].

## 6. Conclusions

Non-alcoholic steatohepatitis is recognised as the cause of NASH-induced cirrhosis. It has also been associated with a significant fraction of cases previously diagnosed as cryptogenic

cirrhosis. Liver cirrhosis can become further complicated by hepatocellular carcinoma, the most frequent primary liver tumour known for serious prognosis and limited treatment options. In addition, the development of HCC in NAFLD patients can precede cirrhosis in a significant fraction of cases. NAFLD is the major hepatic manifestation of obesity and associated metabolic diseases, such as diabetes mellitus. With increasing prevalence of these conditions, NAFLD has become the most common liver disorder worldwide. It affects around 25% of general population and 90% of patients suffering from morbid obesity, i.e., having body mass index equal or greater than 40 kg/m$^2$.

The mechanisms of liver steatosis include up-regulation of inflammatory cytokines, as TNF-$\alpha$, IL-6 and CCL2, released from extrahepatic adipose tissues due to prolonged low-grade inflammation triggered by hypoxia-induced death of fast-growing fat cells. Insulin resistance further contributes to NAFLD and can be aggravated by the pro-inflammatory cytokine background. Free fatty acids and cholesterol cause lipotoxicity due to released reactive oxygen species as well as toxic metabolites generated by non-oxidative biochemical pathways. Decreased level of adiponectin, exaggerated oxidative stress and hepatic iron accumulation also are among the mechanisms of NAFLD.

In the pathogenesis of NAFLD, 20–30% of patients, initially affected by simple liver steatosis, develop hepatic inflammation and thus correspond to the diagnostic criteria of NASH. These cases are at risk to progress to liver cirrhosis and hepatocellular carcinoma. The standardised incidence ratio of HCC in NASH patients reaches 4.4. Regarding the epidemiological profile of hepatocellular carcinoma, the proportion of NASH-related cases is growing and has increased from 8.3 to 13.5% in the time period 2002–2012.

Obesity has been linked to higher frequency of cancers in different organs including the liver. The relative risk of HCC-attributable death in obese patients (body mass index equal or greater than 35 kg/m$^2$) can be as high as 4.5. The underlying mechanisms of carcinogenesis include chronic general low-grade inflammation characterised by elevated levels of TNF-$\alpha$ and IL-6, both of which are tumour-promoting cytokines and major drivers of cell proliferation in NAFLD and NASH. The increased levels of leptin and suppressed production of adiponectin represent another mechanism involved in the progression of NAFLD to HCC in obese individuals. Leptin is a pro-inflammatory, pro-angiogenic and pro-fibrogenic cytokine with a growth-promoting effect. Adiponectin has anti-inflammatory, antiangiogenic and tumour growth-limiting properties. Insulin resistance and hyperinsulinemia lead to excessive cell proliferation. Iron compound deposition has also been related to HCC development in NAFLD-related cirrhosis, possibly due to oxidative DNA damage. Thus, the same molecular pathways that induced NAFLD continue to be active until the development of HCC. These mechanisms are supplemented by critical genetic events including *PTEN* deletion, switch from inactivated to upregulated Wnt pathway and typical mutation pattern in *TP53*. Certain microRNAs, including miR-21, act as molecular switches.

Pathogenetically related molecular markers, e.g., cytokeratin 18, can serve as predictive tests to detect increased risk of HCC.

The molecular pathogenesis of NAFLD is closely related to the selection of treatment targets. NAFLD patients can benefit from low calorie diet, reducing hypertriglyceridemia and potentially reversing steatosis and even fibrosis; physical activity inhibiting mTOR complex;

statins influencing cholesterol synthesis, RAF/MAPK1/ERK and p21 pathway; metformin acting through suppression of mTOR and ROS; pentoxyfillin lowering production of pro-inflammatory cytokines. Multikinase inhibitor sorafenib is indicated in HCC patients. Bidirectional regulation of telomere attrition, senescence, and p21 pathway could be at least theoretically considered in the future. Restoration of wild-type p53 activity becomes possible. The regulation of miRNA machinery also represents a highly attractive future treatment option.

Thus, NAFLD is gaining increasing importance in nowadays medicine as a frequent condition that can lead to such grave complications as liver cirrhosis and hepatocellular carcinoma. Awareness of the molecular profile is helpful to identify the treatment targets and predictive markers.

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Chapter 2

# Noninvasive Diagnostic and Prognostic Assessment Tools for Liver Fibrosis and Cirrhosis in Patients with Chronic Liver Disease

Ju-Seop Kang and Min-Ho Lee

Additional information is available at the end of the chapter

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**Abstract**

Liver fibrosis, that is, excessive accumulation of extracellular matrix protein, occurs and is the wound-healing response and common final pathway of various chronic liver diseases. Advanced hepatic fibrosis caused by chronic liver inflammation eventually progresses to cirrhosis, and prognosis and management of chronic liver diseases depend on the fibrotic severities. Therefore, the early and precise evaluation of severity and status of liver fibrosis provides useful information for diagnosis as well as treatment planning and treatment efficacy and prognosis. Although invasive liver biopsy is the gold standard to assess the nature and severity of hepatic fibrosis, it has several recognized limitations including sampling error and inter-observer variability in interpretation and staging. Furthermore, the dynamic process of fibrosis resulting from progression and regression is difficult to capture with biopsy alone. Therefore, alternative, simple, reliable, and noninvasive direct and indirect serum markers able to predict the presence of significant fibrosis or cirrhosis in patients with chronic liver disease with considerable accuracy were needed. The hepatology experts are actively researching noninvasive methods of fibrosis quantification. The aims of this chapter were to review the nature and limitations of the several noninvasive methods for the assessment of presence and severity of liver fibrosis in patients with chronic liver disease.

**Keywords:** noninvasive method, biomarker, stage of liver fibrosis, cirrhosis, chronic liver disease

## 1. Introduction

Liver fibrosis and cirrhosis are an important and growing global health problem. Patients with non-cirrhotic chronic liver disease may have an increased mortality rate compared to controls [1]. However, mortality and morbidity rates increase exponentially once cirrhosis

develops. Prognosis and management of chronic liver diseases greatly depend on the amount and progression of liver fibrosis. Therefore, the ability to reliably rule out cirrhosis may be considered an important characteristic of any test designed to assess liver fibrosis [2]. The diagnosis of cirrhosis also portends an increased risk of liver-related morbidity [3] as well as mortality [4]. Liver-related mortality and decompensation are expected to continue to increase over the next decade, due to the projected increase in the number of patients with advanced liver fibrosis in the population [5]. Therefore, the accurate and timely evaluation of liver fibrosis is a key step to manage a chronic liver disease and to assess its prognosis and in need of close monitoring, management of complications, and underlying liver disease in patients with advanced stages [6]. For many years, liver biopsy has been considered the "gold standard" for evaluation of liver fibrosis [7]. Pathologists have proposed robust scoring system for staging liver fibrosis such as the semi-quantitative Metavir score (*F*0: no fibrosis, *F*1: portal fibrosis, *F*2: bridging fibrosis, *F*3; bridging fibrosis, and marked, *F*4: cirrhosis) [8] and the modified Ishak score, an expansion of Metavir score [9]. In addition, computer-aided morphometric measurement of collagen-proportional area, a partly automated technique, provides an accurate and linear evaluation of the amount of fibrosis [10]. However, liver biopsy is an invasive procedure with rare but potentially life-threatening complications and prone to sampling errors. Also, liver biopsy gives a snapshot and not an insight into the dynamic changes during the process of fibrogenesis (progression, atatic, or regression). Therefore, liver biopsy has some limitations as follows. First, biopsy is an invasive technique, which has associated morbidity; pain occurs in 20% of patients and major complications such as bleeding or hemobilia in 0.5% [11]. The bleeding rate (0.5%) has not changed significantly in recent years, according to a large multicenter study [12]. The primary factor that appeared to contribute to bleeding risk was platelet count rather than qualitative factors such as operator experience, needle size, or the use of ultrasound to localize the site. Second, the small size of the biopsy makes it prone to sampling variability [13]. Third, the interpretation of the histologic changes can be problematic with inter- and intra-observer variation [14]. These limitations as well as the availability of powerful viral diagnostic tools and new antiviral drugs have rapidly decreased the use of liver biopsy in viral hepatitis and led to the development of noninvasive techniques for the assessment of liver fibrosis. On the other hand, at least some correlation between biopsy stage and outcomes has begun to emerge. In the NIH-HALT C cohort, a correlation was found between the Ishak fibrosis stage and clinical outcomes, the need of liver transplantation, and liver-related deaths in patients with chronic HCV. However, even in this study, up to 25% of the liver biopsy samples were fragmented, which significantly diminished the ability to draw correlations between biopsy findings and clinical outcomes [15]. While some of these methodologies are now generally applied in patients for a top priority of evaluation, biopsy exists within the clinical technique of hepatologists for estimating the causes of complicated diseases or when there are unconformities between clinical characteristics and extents of fibrosis evaluated by noninvasive methodologies [16]. The dynamic process of fibrosis should be best measured as a continuous variable and classical histological staging systems do not permit this [17]. Since liver biopsy is an invasive procedure, cost-intensive, mostly uncomfortable for the patients, and sometimes prone to complication, alternative, simple noninvasive tests have been developed to reliably assess the stage of liver fibrosis. Ongoing efforts include serum markers and imaging based on ultrasound, computed

tomography (CT), and magnetic resonance imaging (MRI). The goal is to develop tests with high specificity and sensitivity to estimate liver fibrosis and predict outcomes [18]. Ideally, noninvasive methodologies of liver fibrosis should be liver specific, easy to perform, reliable, and inexpensive. In addition, it should be accurate not only for the staging of fibrosis but also for the monitoring of disease progression and antiviral therapy efficacy [19]. Scientific attention is currently focused on new antifibrotic therapies, aiming at fibrosis reversibility and cirrhosis regression [20]. It is therefore important, now more than ever, to ensure accurate and prompt assessment of hepatic fibrosis in therapeutic trials of chronic liver disease. Consequently, the demand for noninvasive method substitutes to estimate hepatic fibrosis is a main trial that has provoked research and induced the improvement of noninvasive serological markers of hepatic fibrosis. Several noninvasive serological markers have been described to forecast the existence of significant fibrosis or cirrhosis in patients with chronic hepatic disease with good accuracy. However, most of these markers require complicated calculations, and manipulation in various clinical situations is difficult and inconvenient [21]. Recently, transient elastography (TE, FibroScan) has been introduced as a novel, rapid, noninvasive, and reproducible method to measure liver stiffness [22]. In several studies [22, 23], liver stiffness measurement (LSM) using M probe of FibroScan accurately predicted hepatic fibrosis and cirrhosis in patients with chronic liver disease.

This chapter focuses and provides comparison of invasive and noninvasive methods for assessing the severity of liver fibrosis and aims to provide update on noninvasive diagnostic and prognostic assessment tools for liver fibrosis and cirrhosis in patients with chronic liver disease.

## 2. Mechanism of liver fibrosis

Liver fibrosis is the result of the continuous wound-healing process of the liver to repeated damage [24]. After acute liver injury (e.g., viral hepatitis), parenchymal cells regenerate and replace the necrotic or apoptotic cells. The process is associated with a hepatic inflammatory response and a limited deposition of extracellular matrix (ECM) in the hepatic parenchyma. If the liver injury persists, then eventually the liver regeneration fails, and hepatocytes are substituted with abundant ECM, including fibrillar collagen [25]. This process results in cirrhosis, which can have a bad outcome and high mortality. Progression to this end stage is typically variable but slow, developing over 20–40 years in patients with chronic liver damage; the speed is dependent on both genetic and environmental factors [26]. Liver fibrosis is a common pathological consequence of a variety of chronic stimuli, including viral, alcohol, and autoimmune, drug-induced, cholestatic and metabolic diseases [18, 26–28]. Deposition of excess ECM is rich in fibril-forming collagens [29], which change the normal structure of the liver resulting in pathophysiologic damage to the organ [30]. Liver fibrosis is beneficial at first because it can encapsulate the injury and is considered a reversible process at this stage [31]. In normal liver, ECM is highly dynamic substratum with a precisely regulated balance between synthesis and degradation. Normally, the hepatic ECM comprises less than 3% of the relative area on a liver tissue section and approximately 0.5% of the total wet weight of liver [32]. It is

also a component of Glisson's capsule, portal tracts, central veins, and the subendothelial space of Disse. The most important structural ECM components in liver are collagen, proteoglycans, laminin, fibronectin, and matricellular proteins. The hepatic parenchyma is composed of hepatocytes, endothelial cells, and other resident cells, including hepatic stellate cell (HSCs) and Kupffer cells (KCs). The sinusoid is the hepatic microvascular unit that has an endothelial lining distinguished by fenestration of pores and is separated from the hepatocytes by the space of Disse, where HSCs reside. This space contains a low-density basal membrane-like matrix that is essential for maintaining the differentiated function of parenchymal cell yet is sufficiently porous to enable metabolic exchange between the bloodstream and hepatocytes [26]. During chronic liver injury, however, ECM production exceeds ECM degradation, and liver fibrosis develops as results of the progressive thickening of fibrotic septae and chemical cross-linking of collagen. Moreover, these changes in ECM composition directly stimulate fibrogenesis (**Figure 1**) [33]. After liver injury, disruption of this matrix and replacement by fibrillar collagens I and III and fibronectin have occurred [34, 35]. Fibrosis is characterized histologically and biochemically by a several-fold elevation in the total ECM content of the liver [25].

Accumulation of ECM in the space of Disse leads to loss of the normal fenestrating structures that are characteristic of the endothelial lining, which causes the impairment of the normal bidirectional metabolic exchange between portal blood and hepatocytes. This process is sinusoidal remodeling, termed capillarization of the sinusoid [38]. All major constituents of normal ECM are represented, to some extent, in the newly formed matrix during the fibrogenic process. As in normal ECM, collagen (especially types I and III) and elastin are most abundant proteins, but glycoproteins (fibronectin and laminin) and pure carbohydrates are also present. When compared to normal matrix, scar tissue produced in liver fibrosis has a significantly higher percentage of type I collagen [39]. ECM deposition occurs as a result of an imbalance between excessive ECM production and less degradation. In the normal liver, matrix metalloproteinases (MMPs) have a well-described ECM-degrading function. The activity of MMPs, however, is suppressed in the setting of liver injury as a result of overexpression of tissue inhibitor of metalloproteinase (TIMPs) by the activated HSCs [25]. TIMPs are key regulators of MMPs, by blocking their collagenolytic activity. In addition, TIMP-1 is anti-apoptotic toward HSCs, in part through the induction of Bcl-2, thus promoting the survival of fibrogenic cells [40]. This balance between MMPs and TIMPs is crucial for ECM homeostasis [41]. In human liver, the degree of TIPM-1 expression correlates with the extent of liver fibrosis [42]. In order to preserve matrix homeostasis, ECM also contains MMPs, MMP-1, MMP-8, and MMP-13 that degrade the fibrillary collagen types I and III predominating in fibrosis, while MMP-2 and MMP-9 degrade collagen types IV as well as denatured fibrillary collagens. HSCs are the key source of both MMPs in liver. Although the increase of MMP production should control the excessive increase of the ECM, it can also promote injury. Early increases in MMP, particularly MMP-2, degrade normal matrix and recruit cells that amplify fibrosis [43, 44]. In addition, there is also enhanced secretion of TIMP-1 and -2 by HSCs during progressive tissue injury and cellular activation. Different populations of cells play roles in fibrogenesis, but the activation of HSCs is an essential factor in fibrinogenesis [45]. The mechanism of liver fibrosis is thought to be associated with the hepatic damage of various etiologic

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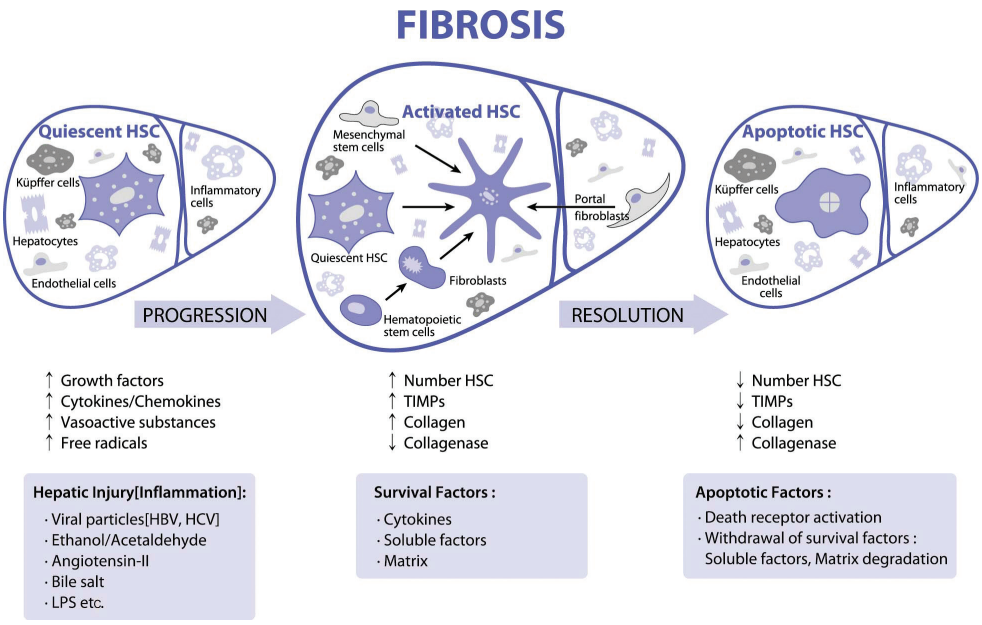


**Figure 1.** Schematic illustration of fibrosis progression and regression (modified from Refs. [18, 36, 37]).

factors followed by the activation of HSCs within the liver that develop into liver myofibroblasts (LMFs) [46]. LMFs include a heterogeneous population of highly proliferative cells that accumulate at injury sites and promote ECM accumulation [47]. The pool of LMF originates mainly from liver mesenchymal cells, namely HSCs [48]. Although HSCs are the primary source of LMFs in liver fibrosis, extrahepatic precursors such as bone marrow-derived mesenchymal cells and portal fibroblasts contribute in ECM production [49, 50]. HSCs are resident peri-sinusoidal cells in the subendothelial space of Disse between hepatocytes and sinusoidal endothelial cells. The main cells affected by liver fibrosis are the HSCs and fibroblasts, which are activated by soluble mediators produced by activated KCs or inflammatory cells in the course of chronic liver disease [51]. ECM may thereby regulate cellular activity and availability of growth factors. For instance, decorin and biglycan, two ECM components, bind transforming growth factor-β (TGF-β), fibronectin and laminin bind tumor necrosis factor-$\alpha$ (TNF-$\alpha$), and collagen binds platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and interleukin-2 (IL-2). The binding of survival factors to the ECM may prevent apoptosis of hepatocyte in the pathologic condition and also prevent growth factor degradation [33]. In liver tissues, HSCs store retinoids such as vitamin A and produce glial fibrillary acidic protein (GFAP), the so-called fat-storing cells or vitamin A-rich cells [52]. Following liver injury, HSCs become activated, which leads to the conversion of a quiescent to activated HSCs that has lost vitamin A droplets, leading to increased proliferation and contraction and the release of proinflammatory, profibrogenic, and promitogenic cytokines. These activated HSCs are capable of enhanced migration and deposition of ECM components [46, 53]. The activation of HSCs can be divided into two stages: initiation and perpetuation [46]. In the first or initiation

phase, HSCs undergo the initial changes toward a myofibroblast-like cell differentiation and become more responsive to proliferative and fibrogenic cytokines by up-regulation of membrane receptors [54]. This stage also called a "pre-inflammatory" stage refers to early changes in gene expression that result primarily from paracrine stimuli derived from damaged resident liver cells (sinusoidal endothelial cells, KCs, and hepatocytes) and platelets. KCs engagement drives release cytokines (especially TGF-β) and ROS signaling [55]. Endothelial cells participate in the conversion of latent TGF-β into active form and produce fibronectin, which also provokes early HSC activation. In addition, PDGF, TGF-β, and endothelial growth factor (EGF) which is potent activators of HSCs [56]. Persistence of these stimuli accompanying sustained injury leads to a perpetuation stage regulated by autocrine and paracrine stimuli. Perpetuation stage involves at least seven distinct changes in HSC behavior, including proliferation, chemotaxis, fibrogenesis, contractility, altered matrix degradation, retinoid loss, and inflammatory signaling [57]. Therefore, a critical event in liver fibrogenesis is that the ECM is a dynamic structure, and even advanced fibrosis may be reversible [58, 59]. Multiple interactions between the ECM, HSCs, endothelial cells, and immune cells have been identified. The central event in liver fibrogenesis appears to be the activation of HSCs, which is a complex process [59]. Different patterns of fibrosis progression have been described on the basis of their etiology, region of injury (e.g., portal or central), the source of fibrogenic cells involved, and the predominant fibrogenic mechanisms [60]. For example, chronic viral hepatitis B and C are major causes of bridging fibrosis, resulting in the formation of portal-central fibrotic septa. Perisinusoidal or pericellular fibrosis is typically found in alcohol-related disorders and nonalcoholic fatty liver disease (NAFLD). Progression of hepatic pathology with sustained fibrogenesis leads to cirrhosis, which is not merely the end-stage accumulation of scar, but rather is characterized by a destruction of the hepatic parenchyma and vascular architecture. The main pathological characteristic of cirrhosis is the formation of nodules of regenerative parenchyma enclosed by fibrotic septa, which may contain terminal hepatic venules and portal tracts when the nodules are especially large (i.e., macronodular cirrhosis). Portosystemic shunts and venous occlusion often occur, leading to impairment in liver function and the development of portal hypertension. The formation of vascularized fibrous septa that link portal tracts and central veins is stimulated by angiogenesis and contributes to portosystemic shunting that bypasses the liver parenchyma [61].

## 3. Liver biopsy: pros and cons, and limitations

Liver biopsy is usually known as the most specific test to evaluate the feature and severity of liver pathology and can be useful in monitoring the efficacy of various treatments. There are currently several techniques available for obtaining liver tissue and each of these has pros and cons [7]. The size of the biopsy specimen, which varies between 10 and 30 mm in length and between 1.2 and 2 mm in diameter, represents only 1:50,000 of the total mass of liver [62]. Therefore, in disease affecting the liver in a diverse way, the histologic findings of biopsy specimen may not be representative of the pathologic process. However, most cases of chronic liver disease causing fibrosis, such as viral and autoimmune hepatitis, as well as nonalcoholic steatohepatitis (NASH), affect the liver in a relatively uniform pattern [63]. Then the extent, to

which the biopsy will be representative, will depend greatly on the size of the specimen obtained. The number of portal triads present in the specimen is important; most hepatopathologists are satisfied with a biopsy specimen containing at least 6–8 portal triads. The indications of liver biopsy are outlined in **Table 1** [7].

| |
|---|
| ◈ Diagnosis, grading, and staging of chronic hepatitis C or chronic hepatitis B. |
| ◈ Diagnosis, grading, and staging of alcoholic liver disease, nonalcoholic steatohepatitis (NASH), or autoimmune hepatitis |
| ◈ Diagnosis of heavy metal storage disorders (e.g., hemochromatosis, Wilson's disease) |
| ◈ Evaluation of the cholestatic liver disease, primary biliary cirrhosis, and primary sclerosing cholangitis |
| ◈ Evaluation of abnormal results of biochemical tests of the liver in association with serological workup that is negative or inconclusive |
| ◈ Use of hepatotoxic regimens (e.g., methotrexate therapy for psoriasis): monitoring |
| ◈ Diagnosis of liver mass (e.g., cancer or unexplained lesions) |
| ◈ Liver donor status before transplantation |
| ◈ Evaluation of systemic illness (e.g., fever of unknown origin, inflammatory or granulomatous disorders) |
| ◈ Hepatosplenomegaly of unknown cause: diagnosis |

**Table 1.** Indication for liver biopsy (modified from Ref. [7]).

Even for patients where serological tests point to a specific liver disease, a liver biopsy can provide valuable information regarding staging, prognosis, and management. There are bad interrelationships between clinical characteristics or status of serum liver enzymes and hepatic histopathologic findings, but also patients with healthy status of liver enzymes may be diagnosed to have clinically advanced fibrosis or cirrhosis on histopathologic findings [64]. If the patient has minor-state illness and is infected with genotype 1a or 1b of the hepatitis C virus, a medical judgment may be made to delay treatment. If the patients have the above degree of moderate disease, treatment will be commonly suggested. If the patients have a virological reaction and acceptable adverse reactions with treatment, continued therapy would be firmly encouraged. The cirrhotic findings on hepatic histopathology will indicate the need for extra tests, such as upper endoscopic procedure to rule out esophageal varices and monitoring for hepatoma with continuing assessment of serum $\alpha$-fetoprotein and hepatic sonography [7]. In alcoholic liver disease (ALD), the grade of the clinical symptoms and the severity of serum liver enzymes elevation correlate poorly with the degree of liver pathology, particularly in patients who continue to consume alcohol. The long-term prognosis depends upon the extent of liver damage [65]. In patients with ALD as well as NASH, liver biopsy may demonstrate hepatic fatty infiltration, ballooning degeneration of hepatocyte, Mallory's bodies, and hepatonecrosis, regardless of clinically severe fibrosis or cirrhosis [7]. In primary biliary cirrhosis (PBC), sequential liver biopsies may assist one to investigate the natural history, track the responses of therapy, or identify a recurrence of the disease after liver transplantation [66, 67]. Liver biopsy allows a precise evaluation in approximately 90% of patients with obscure disorders revealed on liver function tests [68]. The explanation of diverse courses that appear in a transplanted liver including immune reaction, systemic or infectious complications, drug

toxic reaction, and the recurrence of primary disease necessitates a liver histological examination [69]. Liver biopsy can also provide the diagnosis of systemic diseases that can influence the liver, such as sarcoidosis, lymphoma, acquired immune deficiency syndrome (AIDS), and amyloidosis. The histopathological examination of the biopsy material is a subjective process; therefore, diagnostic reproducibility at the 100% level is practically impossible. Intra-observer and inter-observer agreement studies suggest that biopsy specimen size and observer experience (specialization, duration of practice, and academic practice) are important factors in reducing the variation of assessment [8, 70]. Most studies of specimen adequacy have focused on chronic hepatitis because it represents the most common indication for liver biopsy [71]. At present, the most common suggestions for the precise assessment of the degree of fibrosis in chronic hepatic diseases are that the size of biopsy tissue materials must be at a minimum of 20 mm in size and 1.4 mm in radius and must be retained at a minimum of 11 intact portal tracts [72]. In addition, the type of biopsy needle is important, as suction needles tend to miss the fibrous tissue of the septa, as opposed to cutting needles, thus providing the wrong impression regarding the degree of fibrosis and the presence or absence of cirrhosis [73]. Finally, it should be kept in mind that biopsy specimens obtained from subcapsular locations generally contain more fibrous tissue than deeper specimens taken perpendicular to the hepatic surface. For many years, liver biopsy has been considered the gold standard for the staging liver fibrosis [7]. For instance, in patients with chronic HCV, precise definition of the liver fibrosis stage is the important parameter to assess the risk of disease progression and to decide the need for immediate antiviral therapy [74]. Several standardized semi-quantitative scoring systems have been proposed for the staging histological activity index (HAI) proposed in 1981 by Knodell [75] and, more recently, the Ishak score [9] and the Metavir system [76] (**Table 2**). All of these scoring systems have some limitations, being not linear and prone to intra- and inter-observer variation and to sampling variability [77].

The Knodell score is a composite score that is based on histological assessment of periportal and/or bridging necrosis, intralobular degeneration and focal necrosis, portal inflammation, and fibrosis. The score ranges from 0 to 22, with higher scores representing more advanced disease [75]. Knodell score is frequently used in trials of treatments for chronic hepatitis, particularly HCV. The score is used to assure that baseline histologic features in treatment groups are equally matched and to assess histologic changes after therapy. A limitation of the Knodell score is that it combines inflammation and fibrosis to arrive at one composite score, so it is relatively insensitive to changes in fibrosis. This is important because it is fibrosis, and not inflammation per se, that leads to many of the sequelae of chronic liver disease. In addition, patients may have the same Knodell score despite having markedly different degree of fibrosis. Also, the Knodell score is associated with high inter- and intra-observer variability. The Metavir system is a semi-quantitative classification that consists of four intensity degrees of an activity score (A0–A3) and a five-point scale of fibrosis (*F*0–*F*4) [8, 76]. In contrast to the Knodell score, the Metavir system was specifically designed and validated for patients with HCV [76]. The inter- and intra-observer reliabilities of the activity and fibrosis scores of the Metavir system are similar to the Knodell score. The Ishak score is a modification of the Knodell score that includes six stages of fibrosis [9]. This permits documentation of small changes in fibrosis compared with the standard Knodell score, which has only four stages.

| Fibrosis stage | Knodell | Ishak | Metavir | Scheuer | Batt-Ludwig | Laennec |
|---|---|---|---|---|---|---|
| No fibrosis | 0 | 0 | 0 | 0 | 0 | 0 |
| Fibrosis of some portal areas without septa | 1 | 1 | 1 | 1 | 1 | 1 |
| Fibrosis of most portal areas without septa | 1 | 2 | 1 | 1 | 1 | 2 |
| Portal fibrosis with few septa | 3 | 3 | 2 | 2 | 2 | 3 |
| Septal fibrosis without cirrhosis | 3 | 4 | 3 | 2 | 2 | 3 |
| Incomplete cirrhosis | 4 | 5 | 4 | 3 | 4 | 4A |
| Cirrhosis | 4 | 6 | 4 | 4 | 4 | 4B–4C |

**Table 2.** Comparison between three scoring systems for liver fibrosis in chronic viral hepatitis (modified from Refs. [19, 78–80]).

This staging system has become widely used in clinical trials because of its ability to detect mild changes in fibrosis [81]. The Scheuer system is a simple scoring system that separates necrotic inflammation from fibrosis [78]. Histologic findings of portal inflammation, interface hepatitis, and lobular inflammation are each assigned a score of 0–4. A separate score (0–4) is assigned to the stage of fibrosis. Batts-Ludwig system is also known as the modified Scheuer system [79]. This system is applicable to both chronic viral hepatitis and autoimmune hepatitis and is more useful for assessing an individual patient's liver biopsy for clinical care than therapeutic trials. In addition, disease-specific scoring systems are also available, including scoring systems for nonalcoholic fatty liver disease (NAFLD), ALD, primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). However, it is not common to encounter two or more concurrent diseases in a liver biopsy specimen, no scoring systems are available that specifically address these situations [81]. Absolute contraindication to liver biopsy includes patient's inability to remain still and to maintain brief expiration for the procedure, suspected vascular lesion (e.g., hemangioma), bleeding tendency (e.g., INR >1.2 despite receiving vitamin K, bleeding time >10 min), and severe thrombocytopenia (<50,000/mL). Relative contraindications include profound anemia, peritonitis, marked ascites, high-grade biliary obstruction, and a subphrenic or right pleural infection or effusion. Nonetheless, percutaneous liver biopsy is sufficiently safe to be performed on an outpatient setting [82]. Despite liver biopsy being the standard test for an appropriate assessment of patients with chronic liver diseases, there are several limitations of this including variable quality of liver biopsy specimens of <20 mm in length which may be difficult to interpret. Therefore, larger caliber needles may yield better than fine-needle biopsies [71]. Because of fluctuating disease activity, histologic changes obtained at a single point in time may not reflect overall disease activity, which may vary. On the other hand, one would often want to be aware of the progression of liver disease in order to assess therapy response. Limitations entailed by repeat liver biopsy as regards potential patient's risks demand the development of new methods for liver fibrosis evaluation. The features and limitations of liver biopsy are summarized in **Table 3**. On all these grounds, noninvasive diagnostic tests (serum markers and imaging modalities) have been developed of late mainly to assess liver fibrosis severity. The following pages attempt to describe available information on the better-known serum markers as well as imaging techniques.

| | Liver biopsy | Noninvasive methods |
|---|---|---|
| Pros | • Gold standard to assess fibrosis<br>• Direct observation and quantitative assessment of fibrosis, inflammation, and steatosis<br>• Different stage by different scoring systems<br>• Diagnosing different forms of liver disease<br>• Accurately assessing progression of liver disease or the effect of therapy | • Noninvasive<br>• No complications and no contraindications<br>• Inter-laboratory reproducibility<br>• High applicability and wide availability for repeated assays<br>• Reasonable cost<br>• Accurate assessment of cirrhosis and minimal/no fibrosis |
| Cons | • Invasive<br>• Sampling variability/evaluation of a tiny part of the whole organ (1:50,000)<br>• Intra- and inter-observer variability<br>• Unsuitable for repeated assays<br>• Risk of complications, rare major complications, morbidity and mortality<br>• High cost | • Less accurate for intermediate fibrosis stages<br>• False-positive values<br>• Scores may change in different disease stages<br>• Unsuitable for diagnosing liver disease<br>• Not quantitative<br>• "Grey zone" (intermediate results in 14–33% of cases) |

**Table 3.** Pros and cons of liver biopsy and noninvasive methods for the evaluation of liver fibrosis in chronic liver disease (modified from Refs. [83, 84]).

## 4. Noninvasive assessment of liver fibrosis in patients with chronic liver disease

Liver biopsy remains the "gold standard" of assessing hepatic fibrosis. However, it has limitations, such as high cost, invasiveness, associated risk for complications, and sampling or observer variability. Therefore, liver biopsy has recently been challenged by the development of novel noninvasive modalities, including serum direct and/or indirect markers of hepatic fibrosis, noninvasive modalities of predicting fibrosis and imaging techniques, including TE (FibroScan), ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), and thallium 201 per rectal scintigraphy (TI-201 test). As well as TE [85, 86], TI-201 test is a relatively new technique for assessment of liver fibrosis or cirrhosis [87–91]. However, the cost of the equipment may limit the use of TE in some institutions with limited resources. In the past decade, several noninvasive methods for assessing hepatic fibrosis have been published, resulting in more noninvasive tests than histologic scoring systems. The noninvasive tests were introduced to estimate the likelihood of advanced liver fibrosis in patients with chronic viral liver disease at presentation, and on follow-up to assess fibrosis regression in post-treatment period [92]. These tests were later applied in ALD [93, 94] and NAFLD [95, 96]. Our previous studies on the clinical value of the TI-201 test in chronic liver disease may be useful in differentiating chronic hepatitis from cirrhosis and prediction of its prognosis for the management of disease [90, 91, 97, 98]. The first important clinical topic in the assessment of new diagnostic methodologies for evaluation of liver fibrosis is its validation against the present clinical gold standard, liver biopsy, to calculate sensitivity, specificity, and negative- (NPV) and positive-predictive values (PPV). The standard statement of the efficiency of modalities is to examine the area under the receiver operator characteristic curve (AUROC), which plots the sensitivity over 1-specificity using liver biopsy as the reference [17]. The AUROC

| |
|---|
| ◈ Liver specific |
| ◈ Levels not influenced by alterations in liver, renal, or reticuloendothelial function |
| ◈ Measurement of one or more of the following processes: Stage of fibrosis, imbalance of activity of ECM (fibrogenesis vs. degradation) |
| ◈ Easy to perform |

**Table 4.** Features of an ideal marker of liver fibrosis (modified from Ref. [108]).

indicates the probability that a test will correctly rank two randomized patient groups, one with a liver biopsy considered "normal group" and the other "diseased group" [99, 100]. Because liver biopsy itself is not a perfect gold standard, a perfect test will never reach maximal value (1.0) [17]. According to a range of accuracies of the biopsy and a range of prevalence of significant disease (that influence the AUROC), an AUROC of >0.90 in the most favorable scenario cannot be achieved when assessing the so-called "significant fibrosis" even for a perfect marker [99, 101]. This is important for several reasons. First, studies have already shown that these maximal AUROC values have been reached for surrogate markers, especially when assessing cirrhosis versus non-cirrhosis, suggesting that these surrogate markers may be at least as good as liver biopsy in the diagnosis of cirrhosis [102]. Second, some reports suggest that a definitive method for assessing the performance of surrogate markers would employ a clinical end point rather than biopsy as gold standard [101]. The AUROC values may also depend on the biopsy tissue size and fragmentation [103] as well as the incidence of each stage of fibrosis within the studied population (e.g., the spectrum bias) [104]. Indeed, if extreme stages of fibrosis (*F*0 and *F*4) are overestimated in a population, the sensitivity and specificity achieved will automatically be higher than in a population that included only patients with near stages of fibrosis (F1 and F2). Several strategies of prohibiting the "spectrum bias" have been suggested including the realignment of AUROC by the DANA method that define advanced (*F*2–*F*4) and non-advanced fibrosis (*F*0–*F*1) [104] or the Obuchowski measure that is multinomial version of the AUROC [105, 106]. Today, noninvasive methods are widely available. Their most advantages are the absence of contraindication and dangerous complications for the patients, and their reproducibility [107]. In contrast to liver biopsy, many noninvasive methods can effectively evaluate the extent of fibrosis in the whole organ and not only in a part of it. Their potential ability to identify and differentiate between advanced fibrosis stages, the high specificity and sensitivity to diagnose cirrhosis, and their easy application makes them a useful tool in daily clinical practice. Many liver fibrosis experts would therefore consider noninvasive fibrosis tests with an AUROC of 0.85–0.90 to be as good as liver biopsy for diagnosis and staging for liver fibrosis [108]. The role of noninvasive diagnostic tests becomes more significant because their diagnostic accuracy can be increased if they are combined, that is, a serological panel may be used in conjunction with an imaging technique [90, 99, 109]. Features of ideal noninvasive markers of liver fibrosis are summarized in **Table 4**.

### 4.1. Serological markers of liver fibrosis

The clinical need for good noninvasive markers of fibrosis is underlined by the marked increase in the number of reports in this area in recent years. A large number of the serological markers of liver fibrosis have been assessed for the noninvasive evaluation of liver fibrosis and

are broadly categorized into two groups (direct and indirect) [107]. First, we will refer to direct markers of fibrosis that are thought to directly reflect ECM turnover. Fields in which these methods may have clinical or investigational values involve both the noninvasive method for staging of liver fibrosis but they may also be useful for monitoring the behavior of fibrogenesis and ECM metabolism. Therefore, such assays may be valuable in forecasting fibrotic disease deterioration as well as the efficiency of treatment. Second, there are those that reflect changes in hepatic function but do not directly reflect ECM turnover, for instance, platelet count, coagulation studies, and evaluation of liver enzymes, the so-called indirect markers of liver fibrosis. Researches and developments of these markers have largely focused on the diagnosis of cirrhosis, but more recent researches have emphasized the availability of these markers to assess patients with more advanced fibrosis and hence may be valuable in guiding treatment decisions and prediction of complications of liver cirrhosis [90, 108].

*4.1.1. Direct markers of liver fibrosis*

Direct markers of liver fibrosis include serum markers, which have been shown to be, or are thought to be, directly involved in the deposition or degradation of ECM. The best-validated marker is hyaluronic acid (HA), a glycosaminoglycan synthesized by HSCs [110]. HA levels correlate with fibrosis in ALD [111] and chronic viral hepatitis [112, 113] and a highly negative score may be used in clinical practice as a reliable index for exclusion of liver fibrosis. Amino-terminal propeptide of type III collagen is a marker associated with collagen deposition and its level is increased in acute and chronic hepatic diseases [114]. TIMPs (TIMP-1 and -2), on the other hand, are associated with the procedure of collagen degradation, which is progressive to fibrosis consequence [114]. The direct markers include several cytokines and markers of matrix turnover (**Table 5**). The circulating retention times of these molecules are short, so levels may reflect the behavior of ECM turnover. Since ECM turnover is related to both new ECM accumulation and degradation and rebuilding of formed ECM, circulating levels probably exhibit both the activity of the fibrogenesis and the total amount of ECM rebuilding [108]. This phenomenon is identified by at least three properties. First, circulating amounts of these markers are often most increased in situations with rapidly processing fibrosis (e.g., advanced ALD or more active viral hepatitis) and may be high ahead of the significant accumulation of ECM [113, 115]. Second, circulating ECM levels tend toward a decrease in reaction to therapy of the underlying illness, often before any perceptible decrease in the stage of fibrosis [116]. Third, in chronic liver diseases, elevations of several, but not all of these markers associate independently with the stage of fibrosis, rather than with either serological or histopathological findings of inflammatory reaction [112, 117, 118]. In some studies, however, levels of these markers correlated more strongly with the degree of histopathological inflammation or serum liver enzymes [119]. The observation that markers of ECM metabolism are increased in parallel with markers of liver inflammation and necrosis may reflect the importance of these processes in up-regulating fibrogenesis. Direct markers of fibrosis can also be categorized according to their molecular structures. These include (a) collagens: procollagen I and III, propeptides released into the circulation during matrix accumulation and rebuilding. Type IV collagen, which is secreted during interstitial filament metabolism, reflects matrix depletion and rebuilding; (b) glycoproteins and polysaccharides including HA [120], laminin [121], tenascin, and

| Markers of deposition | Markers of degradation | Unknown roles |
|---|---|---|
| ◈ Procollagen I C-terminal | ◈ Procollagen IV C peptide | ◈ Hyaluronic acid(HA) |
| ◈ Procollagen III N-terminal | ◈ Procollagen IV N peptide (7-S collagen) | ◈ Laminin |
| ◈ Tenascin | ◈ Collagen IV | ◈ YKL-40 (Chondrex) |
| ◈ TIMPs | ◈ Undulin | |
| ◈ TGF-β | ◈ MMPs | |

**Table 5.** Direct markers of ECM turnover (deposition vs. degradation) (modified from Ref. [108]).

YKL-40 [122]; and (c) collagenase and their inhibitors, include the MMPs and TIMPs, and cytokines involved in liver fibrosis, the best studies of these is TGF-β. Others, including PDGFs and the antifibrotic cytokine IL-10, have been less well evaluated [108]. The greatest clinical utility of HA may be its ability to exclude patients with significant fibrosis and cirrhosis [112].

*4.1.2. Indirect marker and combined panels of liver fibrosis*

Indirect markers of fibrosis are simple routine blood tests reflecting alterations in liver function but not directly representing ECM homeostasis. These biomarkers include indices related to portal hypertension (platelet count and spleen size), liver synthetic parameters (i.e., albumin), liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [123], AST/ALT ratio [124, 125], bilirubin, prothrombin index (PT) [126], γ-glutamyl transferase (γ-GT), and apolipoprotein $A_1$(apo-$A_1$). They can be used in combination to produce sophisticated serological panels such as PGA index (prothrombin time, γ-GT, and apo-$A_1$) [127–129] and APRI (AST to platelet ratio index) [130, 131]. PGA is one of the first biological indexes used for the noninvasive detection of cirrhosis in ALD patients [127]. APRI is based on serum AST level and platelet [131]. It is calculated as (AST/upper limit of normal*) × 100/platelet count and has been extensively studied in patients with HCV or ALD (*adjusted according to the reference values of each laboratory) [107, 132]. PGA index was subsequently modified to the PGAA index by the addition of $\alpha_2$-macroglobulin which resulted in some improvement in its performance (PGAA) [128]. Analysis of studies of indirect markers of fibrosis reveals several features, which are applicable to routine clinical practice. First, in viral and NAFLD, an AST/ALT ratio of greater than 1 is frequently associated with progressive liver fibrosis or cirrhosis [133–135]. Second, both components of the PGA index such as γ-GT and thrombin index are markers of advanced liver fibrosis and can be used to discern patients with more advanced liver fibrosis. Indeed, the prothrombin index has been carried out alike or better than specific other markers of liver fibrosis [117, 136]. It should be emphasized that these markers represent liver dysfunction or structure rather than the disturbance of normal ECM metabolism (**Table 6**) [108].

*4.1.3. Indices/algorithms combining indirect and direct markers of liver fibrosis*

The limitations of each marker to assess liver fibrosis have led to the development of more sophisticated algorithms or indices combining the results of panels of markers that substantially improved diagnostic accuracy in noninvasive evaluation of liver fibrosis (**Table 7**).

| Direct serum markers | Indirect serum markers/combined panels |
|---|---|
| ◈ HA | ◈ Liver enzymes (ALT, AST) |
| ◈ Laminin | ◈ AST/ALT |
| ◈ YKL-40 | ◈ γ-GT |
| ◈ Procollagen type III | ◈ Platelet count |
| ◈ PIIINP | ◈ Albumin |
| ◈ MMP-1 and -2 | ◈ Bilirubin |
| ◈ TIPMs | ◈ PGA |
| ◈ TGF-β | ◈ APRI |

**Abbreviation**: TIMP, tissue inhibitors of metalloproteinases; MMP, matrix metalloproteinase; ALT, alanine aminotransferase; HA, hyaluronic acid; APRI, aspartate aminotransferase/platelet ratio index; PIIINP, Procollagen III amino terminal; γ-GT, γ-glutamyl transpeptidase.

**Table 6.** Serum noninvasive marker of liver fibrosis (modified from Refs. [19, 84]).

| Scores/algorithms | Description |
|---|---|
| FibroTest [137] | • Most validated algorithm and consider patient age and gender<br>• Five parameters: apo-$A_1$, $\alpha_2$-macroglobulin, γ-GT, total bilirubin, haptoglubin. |
| Hepascore [147] | • Four parameters: bilirubin, γ-GT, HA, TIMP-1, $\alpha_2$-macroglobulin), age, and gender.<br>• Prediction with AUROC 0.81 in significant fibrosis and 0.88 for cirrhosis |
| Fibrospect [148] | • 3 parameters: serum HA, TIMP-1, α2-macroglobulin<br>• Moderate or severe fibrosis versus no fibrosis |
| Fibrometer [149] | • Six parameters: platelet count, prothrombin time, AST, α2-macroglobulin, HA, BUN<br>• Prediction of severe fibrosis in chronic viral hepatitis |
| ViraHep C model [150] | • Probability $= 1/(\exp[-y]) + 1$, $y = -5.17 + 0.2x$race + age(years) + 1.19 × ln (AST, IU/L) –1.76×ln(platelet, $10^3$/mL) + 1.38×ln(AP, IU/L) for severe fibrosis in chronic hepatitis C<br>• Dependent on race (AA, African American = 0, CA, Caucasian American = 1) |
| Glycocirrhotest [151] | • Detection of compensated cirrhosis with 100% specificity and 75% sensitivity.<br>• Follow-up of chronic liver diseases patients without repeated biopsy |
| Fibrosis Probability Index (FPI) [152] | • Multivariate logistic regression analysis identified age, AST, total cholesterol level, insulin resistance (by homeostasis model), and past alcohol intake as independent predictors of significant fibrosis.<br>• 96% sensitivity and NPV 93% at a score of ≥0.2 versus 94% specificity and PPV 87% at a score of ≥0.8.<br>• Probability of significant liver fibrosis in patients with chronic HCV infection and useful guide to make decision for need of biopsy. |
| Goteborg University Cirrhosis Index (GUCI) [153] | • Multivariate logistic regression analysis between fibrosis stage (ref. as Ishak stage)<br>• GUCI formula: normalized AST × prothrombin-INR × 100/platelet count(× $10^9$/L)<br>• 80% sensitivity and 78% specificity for cirrhosis with NPV 97% and PPV 31% |
| Forns score [154] | • 7.811–3.131×ln(PT) + 0.781×ln(γ-GT) + 3.467×ln(age) – 0.014× (cholesterol)<br>• Validation in patients with CHC as well as nonviral chronic hepatitis |

| Scores/algorithms | Description |
|---|---|
| ELF score [155] | • Combination of HA, TIMP-1, amino-terminal propeptide of collagen III collagen.<br>• Useful tool in various chronic liver diseases (e.g,. ALD, NAFLD) |
| APRI+Fibrotest [140] | • Improvement of diagnostic accuracy of Fibrotest for detection of significant fibrosis (≥2 by Metavir) and cirrhosis (F4) in CHC patients.<br>• Accuracy of SAFE biopsy for significant fibrosis and/or cirrhosis: above 90% |
| BAAT score [156] | • Index for NAFLD fibrosis (BMI, age, ALT, TG levels)<br>• 4 features, assigning 1 point for each of the following: BMI ≥28 kg/m$^2$, age ≥50 years, ALT ≥twice the normal values, and TG ≥1.7 mmol/L.<br>• A score of 0 or 1 excludes significant fibrosis with NPV of 100% |
| BARD score [157] | • Combination of three variables (AST/ALT ratio, BMI, Type 2 DM)<br>• (BMI ≥28 = 1, AST/ALT ratio ≥0.8 = 2, diabetes = 1, score ≥2, odds ratio for advanced fibrosis = 17)<br>• The variables such as obesity, diabetes, and age influence the score, resulting in a very low PPV and validated in a cohort of NAFLD |
| NAFLD fibrosis score [158, 159] | • Logistic formula: –1.675+0.037×age(years)+0.094×BMI(kg/m$^2$)+1.13× impaired fasting glucose/diabetes(yes = 1, no = 0)+0.99×AST/ALT ratio-0.013×platelet count(×10$^9$/L)– 0.66 × albumin (g/dL)<br>• Values ≤1.455: no advanced fibrosis vs. ≥0.676: advanced fibrosis |
| FIB-4 score [160] | • 90% NPV in excluding and a satisfying 80% PPV in diagnosing fibrosis.<br>• Calculating formula: (age × AST)/(platelet count(×10$^9$/L) ×$\sqrt{ALT}$<br>• NAFLD score and FIB-4: determination of necessity of liver biopsy in NAFLD |
| *P*-value [98] | • 3 parameters: ALT/AST ratio, prothrombin time, H/L ratio<br>• *P*-value = exp[*y*]/(exp[*y*]+1), *y* = 3.3431–0.8160×ALT/AST–0.343×PT+2.693×H/L ratio<br>• *P* < 7.0: non-cirrhotic patients (96.2%) |

**Abbreviation**: AP, alkaline phosphatase; NPV, negative-predictive value; PPV, positive-predictive value, CHC, BMI, body mass index; H/L ratio, heart/liver uptake ratio; γ-GT, γ-glutamyl transpeptidase.

**Table 7.** Combined scores/algorithms for evaluation of liver fibrosis.

In most studies, indices have been validated against the current clinical gold standard, liver biopsy, using as expression of their effectiveness the AUROC with optimal value being as close as possible [99]. The first proposed index was based on a parented mathematical formula combining five variables (total bilirubin, γ-GT, haptoglobin, α2-macroglobulin, and apo-$A_1$) [137] and the results of this test were ranged from 0 to 1.0, using Fibrotest as a reference. In the initial report, a very low score (<0.1) allowed the exclusion of significant fibrosis with a 100% negative-predictive value (NPV), whereas a moderate high score (>0.6) allowed the diagnosis of significant fibrosis with a 90% positive-predictive value (PPV), using liver biopsy as a reference. Overall, liver biopsy could have been avoided in 46% of the patients from that study. Fibrotest has been primarily used for patients with chronic viral hepatitis and is now extensively evaluated in the patients with chronic hepatitis C [109, 138, 139] but also in other cases, such as hepatitis B [140, 141], HCV and HIV coinfection [142], NAFLD [143], ALD [93], and renal-transplanted patients with chronic HCV [144]. The recent meta-analysis that pooled 7985 subjects (with analysis of individual data in 3282) with both Fibrotest and biopsy (HCV, 4600; HBV, 1580; NAFLD, 267; ALD, 524; mixed form, 1014) and the mean standardized AUROC for

diagnosing significant fibrosis was 0.84 (95% confidence interval: 0.83–0.89), without differences between causes of liver disease. Therefore, Fibrotest have been used as an alternative to liver biopsy for the first-line assessment of fibrosis and common chronic liver diseases, namely HCV, HBV, NAFLD, and ALD [145]. One of the important issues of these algorithms is that in individual patients they cannot reliably differentiate the intermediate stages of fibrosis. Finally, in patients with chronic HCV, the application of these algorithms or indices can confirm or exclude fibrosis in less than 40% of patients [146].

*4.1.4. Combination of indices and algorithms for assessment of liver fibrosis*

In order to increase diagnostic accuracy, new approaches using stepwise algorithms combining continually different indices have been proposed in patients with chronic hepatitis C [161] and B [140]. For instance, one group was able to identify significant fibrosis with high diagnostic outcome above 94% diagnostic accuracy by APRI as screening procedure, followed by Fibrotest in APRI non-classified cases and prohibiting liver biopsy to patients classified *F*0–*F*1 by noninvasive procedures. Cirrhosis could also have been recognized with 95% diagnostic accuracy applying a similar algorithm by the combination of APRI and Fibrotest (**Figure 2**). On the whole, liver biopsy could have been prevented in approximately 50 and 80% of patients for the diagnosis of severe fibrosis and cirrhosis in patients with chronic hepatitis C, respectively. Other groups have proposed alternative algorithms combining Fibrotest and APRI either with [162] or without Forns index [146]. Otherwise, high diagnostic accuracy for the evaluation of significant fibrosis and cirrhosis has been reported for the combination of Fibrotest with Fibroscan that is based on the assessment of hepatic stiffness by TE [22, 109]. However, this method requires the availability of complex equipment, with limited access and costs that most likely exceed those of their more simple and accessible algorithms [161].

Several noninvasive markers of liver fibrosis have been represented but their application in substitute for liver biopsy may still remain controversy and is not generally acceptable due to still insufficient diagnostic performance. In fact, some of these methodologies such as APRI and Forns index remain in many cases unclassified group and all of them are not over 80–85% diagnostic performance [131, 139, 163, 164]. As a consequence, many patients still need to have a liver biopsy taken, and in those classified without liver biopsy, misdiagnosis is expected to occur in at least 15–20%, a figure that is considered inadequate by many clinicians [165, 166]. Most of them, such as APRI and Forns index, are not able to identify individual stages of fibrosis. APRI cannot be completely standardized due to the variability of measurement and normal ranges of AST in different laboratories [167]. Since the diagnostic performance of described noninvasive markers is variable depending on the stage of fibrosis and other patients' characteristics, they can be used to reduce rather than completely substitute the need for liver biopsy. Even though many studied have been shown that Fibrotest had the best performance when compared to other noninvasive methods, none of the investigated noninvasive markers of liver fibrosis has adequate accuracy for universal use instead of liver biopsy [140]. And, one of the major critical points of the clinical application of serum markers and indices of liver fibrosis is that they are not regularly useful in most clinical situation. Another clinical point of these markers is that they are liver nonspecific and may be influenced by changes of their level; for example, HA levels increase after the meal [168] or in senile

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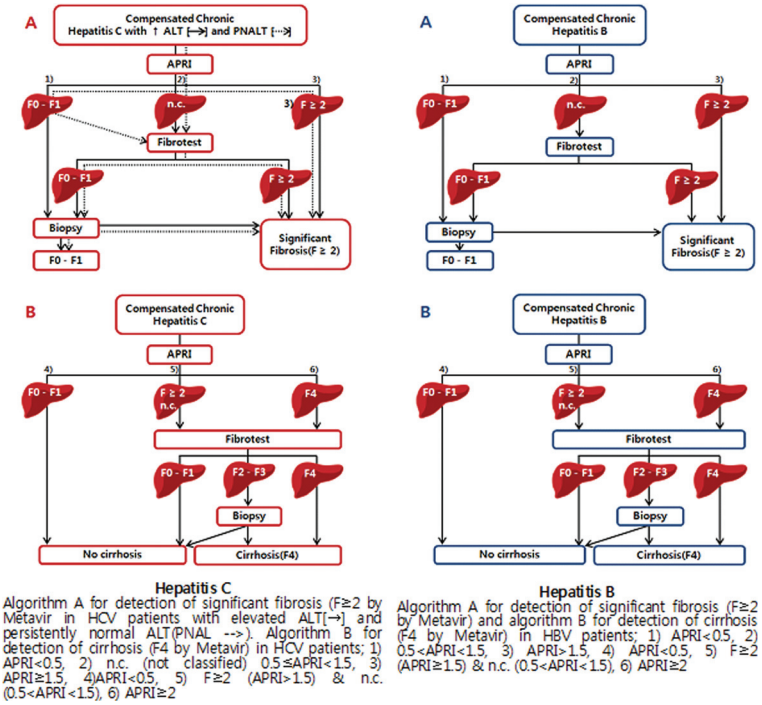


**Figure 2.** Proposed best algorithm for the detection of significant fibrosis and cirrhosis using APRI and Fibrotest in patients with chronic hepatitis C and B with diagnostic accuracy (modified from Refs. [90, 159]).

patients with chronic inflammatory states such as rheumatoid arthritis [169]. Also, the repeatability of assessments of several biomarkers included in direct serum markers, such as AST levels or platelet count, is doubtful [170]. The effect of serum lipid levels caused by anticholesteremic agents on the Forns index was taken into consideration. Finally, when applying Fibrotest in clinical situation, the evaluation should consider each of the five markers individually in order to escape false-positive outcomes related to hemolysis (low hepatoglobin level), Gilbert syndrome (high bilirubin level), or false-negative outcomes related to inflammatory reactions [171]. However, a panel that combines proteins and proteinases of the ECM has been proposed and the results are promising [155]. The combined use of some of these markers with the aim of reducing rather than completely abolishing liver biopsy may represent a rational and more convincing approach [172]. In a large-scale multicenter study, the diagnostic accuracy of a stepwise combination of two well-studied noninvasive markers of fibrosis (APRI and Fibrotest) was followed by liver biopsy in only a subset of cases [171]. This approach, called SAFE (sequential algorithm for fibrosis evaluation) biopsy, has been built up with double goal of detecting both severe fibrosis and cirrhosis and has here been confirmed to assure >90% diagnostic accuracy in comparison with respect to liver biopsy as the gold standard with <2% underestimation of the stage of liver disease as derived from NPV. The

SAFE biopsy may be particularly useful for screening HCV-infected patients in whom an immediate approach with liver biopsy is particularly problematic or questionable [173]. Using two algorithms (Fibrotest and APRI), liver biopsy could be avoided in 50% of cases for the diagnosis of significant fibrosis and in 70% of cases for the identification of cirrhosis [174].

### 4.2. Imaging modalities and combinations with other markers for the diagnosis of liver fibrosis

#### *4.2.1. Transient elastography (TE)*

Liver fibrosis can be staged using one-dimensional ultrasound TE (Fibroscan) [22], which is the most widely used imaging method for noninvasive and rapid measurement of hepatic tissue stiffness. Many studies have evaluated the diagnostic accuracy of TE for diagnosing cirrhosis with specificity and sensitivity approaching 90%. The accuracy for liver fibrosis detection is lower, with sensitivity and specificity approaching 70–80% [102, 175, 176]. Because both adipose tissue and the presence of fluid may influence the velocity of shear wave [107], obesity, ascites, acute inflammation, liver congestion, and elevated portal vein pressure may reduce TE accuracy. Furthermore, a falsely increased liver stiffness, due to postprandial increase in portal vein pressure, has been observed [177, 178]. Comparison of TE with biopsy results has provided that cut-off values can be demonstrated to differentiate mild and moderate fibrosis from advanced fibrosis and cirrhosis, with validation tests showing variable performance and with greatest statistical significance being ensured in the distinction of cirrhosis from mild fibrosis (AUROC $F = 4$ (0.94), sensitivity $F \geq 2$ (85%), specificity $F \geq 2$ (91%)) [179, 180]. Investigations have applied various best stiffness cut-off values, making comparison between researches. Generally, advanced fibrosis is more likely with higher cut-off values (**Table 8**) [181, 182]. The optimal cut-off value is 14.6 kPa for the detection of cirrhosis, but a cut-off value of 10.0 and 14.1 kPa was adequate to achieve 95% sensitivity and specificity in their HCV patients with cirrhosis [183]. Otherwise, the performance of TE was low for discriminating mild from significant liver fibrosis [184] and Spearman's correlation coefficient between the elasticity scores using real-time TE and histopathological fibrosis stage was low at 0.48 [185]. However, TE was more useful for the identification of advanced fibrosis and their necroinflammatory activity influences TE measurements in patients without cirrhosis [186] and might be overestimated liver fibrosis when ALT is elevated [187]. Some reports were shown that good correlation between TE and fibrosis exists, but data on TE in an Asian cohort show only 8% of patients having limited HCV [188]. Liver stiffness measurement (LSM) by TE is a reliable predictor of liver fibrosis in Indian patients with chronic hepatitis C and B. LSM is superior to APRI for noninvasive diagnosis of hepatic fibrosis and cirrhosis, and high bilirubin (10.5 mg/dL) and Ishak HAI grade (>11) were independent predictors of discordance between liver biopsy and LSM [189]. Liver stiffness has also been revealed to have good correlation with steatosis, necrotic inflammatory activity and hepatic iron accumulation as well as fibrosis [190]. TE is restrictive, however, by its impossibility to perform in patients with ascites and patients with narrow intercostal spaces or morbid obesity. Advantages of TE include a short procedure time (<5 min), immediate results, and the ability to perform the test at the bedside or in an outpatient clinic.

| Etiologies | Patients (*n*) | Metavir score | | Cut-offs (kPa) | AUROC | SE (%) | SP (%) | CC (%) |
|---|---|---|---|---|---|---|---|---|
| | | *F* ≥ 2 (%) | *F* 4 (%) | | | | | |
| HCV [109] | 183 | 74 | | 7.1 | 0.83 | 67 | 89 | 73 |
| | | | 25 | 12.5 | 0.95 | 87 | 91 | 90 |
| HCV [181] | 251 | 65 | | 8.6 | 0.79 | 56 | 91 | 68 |
| | | | 19 | 14.6 | 0.87 | 86 | 96 | 94 |
| HCV [186] | 150 | 56 | | 7.8 | 0.91 | 83 | 82 | 83 |
| | | | 19 | 14.8 | 0.98 | 94 | 92 | 92 |
| HCV [190] | 324 | 65 | | 7.4 | 0.86 | 76 | 84 | 79 |
| | | | 21 | 11.9 | 0.94 | 87 | 91 | 90 |
| HBV(CV)[179] | 228 | 62 | | 8.3 | 0.93 | 90 | 32 | 57 |
| | | | 50 | 14.0 | 0.96 | 78 | 98 | 88 |
| HBV [192] | 173 | 50 | | 7.2 | 0.81 | 70 | 83 | 76 |
| | | | 8 | 11.0 | 0.93 | 93 | 87 | 94 |
| HBV [193] | 284 | 42 | | 5.2 | 0.78 | 89 | 38 | 59 |
| | | | 10 | 12.9 | 0.85 | 52 | 93 | 89 |

**Abbreviations:** AUROC, area under the receiver operator characteristic curve; CC, correctly classified: true positive and negative; HBV, chronic hepatitis B; HCV, chronic hepatitis C; SE, sensitivity; SP, specificity.

**Table 8.** Diagnostic performance of TE for significant fibrosis (*F* ≥ 2) and cirrhosis (*F*4) in patients with Hepatitis B or C (modified from Refs. [191]).

### *4.2.2. Magnetic resonance elastography (MRE)*

MRE is a noninvasive method of measuring the viscoelastic properties of the liver and evaluate liver stiffness by measuring the propagation of mechanical waves [194]. MRE indicated that patients with hepatic fibrosis have higher LSM than normal volunteers [195] and that those with mild fibrosis were able to be distinguished from those with moderate or advanced fibrosis, with a mean hepatic shear elasticity being 2.24, 2.56, and 4.68 kPa in patients with *F*0–*F*1, *F*2–*F*3, and *F*4 fibrosis, respectively [196]. MRE is superior to TE because of its ability to scan the whole organ and its application in patients with ascites or obesity. MRE was accurate in liver fibrosis staging and superior to biochemical testing with APRIs in patients with chronic HBV and HCV infection [197, 198]. These findings suggest that noninvasive MRE potentially has a role in determining the treatment and the prognosis of patients with chronic liver disease because it enables substantial and advanced fibrosis to be readily diagnosed. More particularly, MRE might be useful in the selection of patients with liver fibrosis who should either be treated (score of ≥*F*2) or undergo surveillance for portal hypertension and hepatocellular carcinoma (score of ≥*F*3) [197]. Antiviral treatment should be considered in patients with liver stiffness values of ≥2.8 kPa [199]. The main drawbacks are the high cost and complexity of the method that is too procrastinating for daily clinical

practice. MRE values may be affected by the increased portal vein pressure following a meal similar to TE [200].

*4.2.3. Acoustic radiation force impulses (ARFI)*

ARFI use conventional hepatic ultrasonography to assess liver stiffness [199, 201]. ARFI uses short duration of acoustic pulses that produce mechanical excitation. The speed of the produced waves correlates directly with the extent of liver fibrosis and results are expressed in m/s. For fibrosis quantification, the "Virtual Touch (VT) tissue quantification" application was used, allowing for the measurement of SWV (shear wave velocity, m/s) within the interest area chosen by the examiner, according to principles. The higher the tissue stiffness shows, the higher the SWV produces [202]. The theoretical advantage of ARFI as compared to TE is its implementation on an ultrasound device, via additional software imaging control and detection algorithms, thus allowing the visualization of B-mode, color Doppler mode, and ARFI images with same equipment [201]. Advantages of this technology include the ability to select the area to be assessed, avoiding large vessels or ribs [107] and the fact that steatosis does not influence the accuracy of the procedure. Otherwise, ARFI and TE are influenced by high ALT levels. In European patients with chronic hepatitis B and C, ALT values between 1.1*x* and 5*x*ULN had only limited influence on ARFI values. The best cut-off values for predicting significant fibrosis and cirrhosis were similar in patients with moderately elevated ALT levels [203].

*4.2.4. Real-time sonography-based elastography (RTE)*

RTE is a new method for the measurement of tissue elasticity different from TE. The echo signals are captured in the real time, while the probe slightly compresses or relaxes the body through freehand operation. Many clinical researches indicated that RTE could allow a high accuracy on the differential diagnosis of superficial focal pathological lesion such as mammary gland tumors, thyroid tumors, and prostate tumors [204, 205]. This method estimates the velocity of a shear wave through the liver using US and results are expressed in kPa. The diagnostic accuracies expressed as AUROC were 0.75 for the diagnosis of significant fibrosis ($F \geq 2$), 0.73 for severe fibrosis ($F \geq 3$), and 0.69 for cirrhosis. For a combined elasticity-laboratory scores (platelet count and $\gamma$-GT), AUROCs were 0.93, 0.95, and 0.91, respectively. Therefore, RTE is a new and promising sonography-based noninvasive method for the assessment of liver fibrosis in patients with chronic viral hepatitis [185].

*4.2.5. 2D-Shear wave elastography (2D-SWE)*

2D-SWE combines ultrasound images with radiation force induced into the liver. 2D-SWE can measure shear waves propagation in real time [16]. Advantages of 2D-SWE (m/s or kPa) include good applicability and adjustable region of interest depending on the operator [84]. Its failure rate is significantly lower than that of TE [206–208], particularly in patients with ascites [207, 208], but not in obese patients when the XL probe is used for TE (10.4 vs. 2.6%, respectively) [209]. In a pilot study in 121 patients with chronic hepatitis C (Metavir, 41% *F*0/*F*1, 27% *F*2, 12% *F*3, and 20% *F*4), AUROCs of 2D-AWE for the diagnosis of significant fibrosis

and cirrhosis were 0.92 and 0.98, respectively [206]. Sensitivities and specificities were 85 and 92% for the diagnosis of significant fibrosis using a cut-off of 7.1 kPa, and 97 and 93% for the diagnosis of liver cirrhosis using a cut-off of 10.1 kPa. Therefore, 2D-SWE is a promising technique that is currently under investigation. It seems to be at least equivalent to TE and pSWE/ARFI for noninvasive staging of liver fibrosis in viral hepatitis [16].

#### *4.2.6. Sonography-based imaging*

US imaging has been used to noninvasively evaluate the severity of liver fibrosis in patients with chronic HCV. Results vary with some studies showing associations between US score and diagnosis of cirrhosis with various sensitivities (87.5–100%) and specificities (81.5–93.5%) [210, 211]. The application of US to assess liver fibrosis was used by calculating a fibrosis extraction ratio (FER) (fiber volume/total volume), which was able to distinguish *F*0/*F*1 from ≥*F*2 fibrosis with a sensitivity of 55% in the HCV cohort [212]. In sonography, contrast-enhanced sonography is based on intravenous injection of specifically sized microbubbles, transferred with a shell of protein or biopolymers that facilitate their sonographic imaging [213]. Some report studied the hepatic vein transit time (HVTT) for grading liver disease using a microsound microbubble contrast agent as a tracer. This study also applied Doppler sonography to make a decision for several indices to assess portal vein congestive index, but found that there was no significance. HVTT was significantly shorter in cirrhotic patients than in non-cirrhotic patients ($p < 0.001$) and distinguished between these patients with high accuracy [214]. Therefore, unenhanced Doppler ultrasound is not reliable in the discrimination of varying degrees of fibrosis, but that results can be improved with additional measurement such as heart pulsation at the liver surface and portal venous flow measurements. Color Doppler is a noninvasive method for assessing portal hemodynamics. In the study for portal hemodynamics by color Doppler and gastric mucosal blood flow (GMBF) by laser Doppler velocimetry in patients with cirrhosis, portal venous blood flow (PVBF), portal flow velocity (PFV), and GMBF were all significantly slower in cirrhotic patients and PVBF and PFV were lower in Child's class B/C than in class A [215]. A statistically significant difference has been shown in all US markers between patients with and without cirrhosis, but sensitivity and specificity were significantly increased when evaluation of the transmission of heart pulses on the liver surface area included as part of the US test a sensitivity of 85 versus 55% and a specificity of 93 versus 86%, respectively [216].

#### *4.2.7. Per rectum TI-201 scintigraphy (TI-201 test)*

A complete understanding of the hepatic disease requires the evaluation of portal circulation, which allows for more appropriate treatment and follow-up of patients. During the last six decades or more, several clinical reports have investigated portal circulation by radioactive tracers [87, 88]. These reports have been established that TI-201 test allows us to understand the portal circulation, and a new method using TI-201 distribution patterns seems to be useful in evaluating the portosystemic shunt (heart/liver uptake ratio, H/L ratio), which can develop to varying extents in liver cirrhosis and positive correlation to portal pressure in patients with chronic hepatitis [87–89, 217]. Our previous studies on the clinical value of H/L ratio in chronic liver disease may be useful in differentiating chronic hepatitis from cirrhosis and the prediction

of its prognosis for the management of disease [97]. Noninvasive test such as maximal removal rate of indocyanine green and H/L ratio, as well as ALT/AST ratio, prothrombin time, and platelet count, may be used to evaluate the progression of chronic liver disease without liver biopsy [98] as well as progression of variceal bleeding without an endoscopy in biopsy-proven patients with cirrhosis [218]. However, because most serum markers except H/L ratio may be changeable by medical treatment of chronic liver disease, serum markers are not suitable for monitoring long-term outcomes of patients with cirrhosis. On assessing the predictive values of H/L ratio for decompensation during the follow-up period of 45.5 months in 107 patients [90], the last visiting value of H/L ratio provided a strongly reliable predictor of decompensation with an odds ratio estimate of 14.4, an AUROC of 0.825, a cut-off of 0.4, a sensitivity of 73.1%, and a specificity of 71.6% (**Figure 3**).

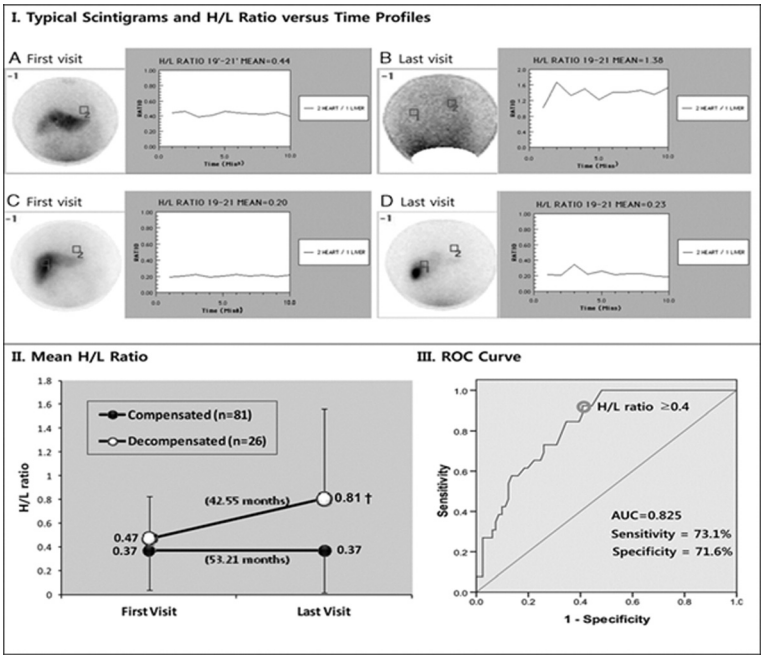


**Figure 3.** I. Typical scintigrams after administration per rectum of 18.5 MBq 201TI and H/L ratio versus time profiles in a decompensated patient (A, B) and compensated patient (C, D) at the first and last visits. On the left I (AYD) are scintigrams after administration per rectum of 18.5 MBq 201TI in each patient (ROI g1, liver area vs. ROI g2, heart area) and on the right I (AYD) are time-activity curves for the H/L ratio in each patient. ROI, regions of interest. II. Mean H/L ratio of the first visit when the patient is diagnosed with cirrhosis and the last visit before the development of decompensation in patients with liver cirrhosis. III. ROC curve and cut-off point of last visit H/L ratio (Q0.4) (permission from Ref. [90]).

## 5. Conclusion and perspectives

There is an urgent need to pursue the development of noninvasive tests in addition to a liver biopsy for the staging of fibrosis. The area of liver fibrosis and cirrhosis has been extensively

studied during the few decades. As a result of growing understanding of liver injury and fibrosis, a number of noninvasive tests for fibrosis that are accurate and replace liver biopsy are being used to develop, commercialized, and are being used more and more in practices. The current serum tests are a start and may have utility in identifying patients with minimal fibrosis who do not require a liver biopsy. Because of the conditional relationship with biopsy, the development of serum markers will always have obvious limitations. The use of noninvasive tools varies widely depending on practice setting and the individual physician's management style. However, as with many new diagnostic methodologies, such tests are being adopted and marketed while the evidence of their general usefulness in various clinical settings remains incomplete. For instance, there is no solid evidence that the currently available tests for liver fibrosis have the precision necessary for tracing disease progression in real time or patient's response to therapy. Before such tests are accepted, their superiority to routine laboratory studies should be demonstrated. Although invasive liver biopsy is still the gold standard to assess the nature and severity of hepatic fibrosis, it has several recognized limitations including sampling error and inter-observer variability in interpretation and staging. Furthermore, the dynamic process of fibrosis resulting from progression and regression is difficult to capture with biopsy alone. Therefore, alternative, simple, reliable, and noninvasive direct and indirect serum markers able to predict the presence of significant fibrosis or cirrhosis in patients with chronic liver disease with considerable accuracy were needed. The hepatology experts are actively researching noninvasive methods of fibrosis quantification. This chapter reviewed the nature and limitations of the several noninvasive methods for the assessment of the presence and severity of liver fibrosis in patients with chronic liver disease.

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Chapter 3

# Alternative Diagnostic Tests of Gastroesophageal Varices in Liver Cirrhosis: Recent Advance

Xingshun Qi, Qiang Zhu and Ye Tian

Additional information is available at the end of the chapter

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**Abstract**

Routine screening for gastroesophageal varices in liver cirrhosis is necessary. At present, upper gastrointestinal endoscopy is the golden diagnostic test of gastroesophageal varices. However, the use of upper gastrointestinal endoscopy is restricted because of its poor compliance and adverse events. In this chapter, we reviewed the recent evidence regarding the value of noninvasive or less invasive tests for the diagnosis of gastroesophageal varices in liver cirrhosis.

**Keywords:** varices, liver cirrhosis, endoscopy, meta-analysis, noninvasive

## 1. Introduction

Gastroesophageal varices and their related bleeding are one of the most common and lethal complications of liver cirrhosis [1, 2]. The prevalence of gastroesophageal varices is approximately 50% at the diagnosis of liver cirrhosis [2]. In the absence of any interventions, Groszmann et al. reported that the incidence of confirmed small varices, large varices, and variceal bleeding in patients without any previous history of varices was 28.6, 3.8, and 2.9% during a median duration of follow-up of 54.9 months, respectively [3]. Merli et al. reported that the 1-, 2-, and 3-year incidence of varices in cirrhotic patients without varices was 5, 17, and 28%, respectively [4]. In this chapter, we mainly review the following contents: the practice guideline and consensus recommendations regarding screening for gastroesophageal varices in liver cirrhosis, current understanding regarding alternative diagnostic tests of gastroesophageal varices in liver cirrhosis, and diagnostic accuracy of different alternative diagnostic tests.

## 2. Screening for gastroesophageal varices in liver cirrhosis

Upper gastrointestinal endoscopy is the golden diagnostic test of gastroesophageal varices. There are some recommendations from practice guideline and consensus regarding endoscopic screening for gastroesophageal varices in liver cirrhosis.

According to the UK practice guideline on the management of variceal hemorrhage in cirrhotic patients, there are high levels of evidence regarding the surveillance of gastroesophageal varices in liver cirrhosis [5]. First, all patients with cirrhosis should undergo endoscopy at the time of diagnosis. Second, in the absence of varices, patients with cirrhosis should undergo endoscopy every 2–3 years. Third, in the cases of grade I varices, patients with cirrhosis should undergo endoscopy every year. Fourth, in the cases of disease progression, the intervals of endoscopy can be modified by the clinicians.

According to the Baveno VI consensus workshop, there are low levels of evidence and weak grade of recommendation regarding the surveillance of esophageal varices in liver cirrhosis [6]. First, compensated cirrhosis without ongoing liver injury or varices should undergo endoscopy every 3 years. Second, compensated cirrhosis with ongoing liver injury without varices should undergo endoscopy every 2 years. Third, compensated patients with small varices without ongoing liver injury should undergo endoscopy every 2 years. Fourth, compensated patients with ongoing liver injury and small varices should undergo endoscopy every year.

The recommendations of the 2016 Practice Guidance by the American Association for the Study of Liver Diseases are similar to those of the Baveno VI consensus [7]. First, in the absence of varices, compensated cirrhosis with and without ongoing liver injury should undergo endoscopy every 2 and 3 years, respectively. Second, in the presence of small varices, compensated cirrhosis with and without ongoing liver injury should undergo endoscopy every 1 and 2 years, respectively. Third, compensated cirrhosis should undergo endoscopy at the time when decompensation events develop.

Although the recommendations regarding the interval of endoscopy and target population are heterogeneous among practice guidelines, repeated endoscopy is necessary for cirrhotic patients. However, endoscopic examinations have several limitations. First, nearly all patients are reluctant for endoscopy. Patients may have poor complaint regarding endoscopy. Second, not all endoscopic examinations are safe. The endoscopy-related adverse events are more frequent and severe in patients with cardiovascular and cerebrovascular diseases.

## 3. Current knowledge about alternative diagnostic tests of gastroesophageal varices in liver cirrhosis

A questionnaire survey assessed the knowledge about alternative diagnostic tests of gastroesophageal varices in 42 members from the Gastroenterology Branch of the Liaoning Medical Association, China [8]. Indeed, alternative diagnostic tests are rarely or never employed in

clinical practice. In the following text, several major alternative diagnostic tests, such as serum liver fibrosis parameters, platelet count to spleen diameter ratio (PSR), liver and spleen stiffness, capsule endoscopy, and computed tomography, are reviewed on the basis of major evidence, especially the results of meta-analyses. The data regarding sensitivity and specificity are primarily presented.

## 4. Serum liver fibrosis parameters for diagnosis of gastroesophageal varices

Hyaluronic acid (HA), laminin (LN), amino-terminal propeptide of type III procollagen (PIIINP), and collagen IV (CIV) are major serum parameters for the assessment of liver fibrosis. A retrospective study evaluated their value of diagnosis of gastroesophageal varices [9]. Unfortunately, all of them could not accurately predict the presence of gastroesophageal varices.

APRI, AAR, FIB-4, FI, King, Lok, Forns, and FibroIndex are the major scores for the assessment of liver fibrosis. Deng et al. systematically reviewed their diagnostic accuracy of gastroesophageal varices [10]. The authors found that APRI, AAR, FIB-4, Lok, Forns, and FibroIndex scores had been evaluated, but not FI or King score. As for the diagnosis of gastroesophageal varices, the sensitivity and specificity of APRI were 0.60 and 0.67, respectively; those of AAR were 0.64 and 0.63, respectively; those of Lok were 0.74 and 0.68, respectively; and the area under the summary receiver operating characteristic curve of these scores ranged from 0.6774 to 0.7885. As for the diagnosis of large varices, the sensitivity and specificity of APRI were 0.65 and 0.66, respectively; those of AAR were 0.68 and 0.58, respectively; those of FIB-4 were 0.62 and 0.64, respectively; those of Lok were 0.78 and 0.63, respectively; those of Forns were 0.65 and 0.61, respectively; and the area under the summary receiver operating characteristic curve of these scores ranged from 0.6530 to 0.7448. More recently, a retrospective study further confirmed these findings [11]. More importantly, their diagnostic accuracy should be improved after the exclusion of previous gastrointestinal bleeding and splenectomy.

## 5. PSR for diagnosis of gastroesophageal varices

PSR is a ratio of the platelet count (/mm$^3$) to the spleen diameter (mm). Multiple meta-analyses evaluated the diagnostic accuracy of PSR for varices. Chawla et al. conducted a meta-analysis of eight studies to explore the diagnostic accuracy of PSR with a cut-off value of 909 for the presence of esophageal varices in cirrhosis [12]. They found a sensitivity of 0.89 and a specificity of 0.74, but the evidence was of low quality according to the GRADE rule. Ying et al. performed another meta-analysis of 20 studies to assess the value of PSR with a cut-off value of 909 for esophageal varices in cirrhosis [13]. By comparison, they showed a relatively higher sensitivity of 0.92 and a specificity of 0.87, and the quality of studies was moderate according to the quality assessment of diagnostic accuracy studies (QUADAS) questionnaires. More recently, Chen et al. reported the results from an updated meta-analysis of 49 studies that the

summary sensitivity and specificity of PSR for any varices were 0.84 and 0.78, respectively, and that the summary sensitivity and specificity of PSR for high-risk varices were 0.78 and 0.67, respectively [14]. Similarly, the authors considered that the quality of included studies was moderate. Taken together, the evidence supported the use of PSR for identifying the presence of varices. However, its diagnostic accuracy is not high.

## 6. Liver and spleen stiffness measurement for diagnosis of gastroesophageal varices

Major evidence can be obtained from the results of several large meta-analyses. Pu et al. identified a total of 15 papers regarding liver stiffness measurement by FibroScan transient elastography for esophageal varices [15]. The pooled sensitivity and specificity of liver stiffness for any varices were 0.84 and 0.62, respectively; the pooled sensitivity and specificity of liver stiffness for large varices were 0.78 and 0.76, respectively. Similarly, Qu et al. also performed a meta-analysis of 20 studies to evaluate the performance of liver stiffness by transient elastography for esophageal varices [16]. As for any varices, the pooled sensitivity and specificity were 0.84 and 0.68, respectively. As for large varices, the pooled sensitivity and specificity were 0.84 and 0.72, respectively. Singh et al. synthesized the data from 12 studies regarding spleen stiffness for the diagnosis of esophageal varices [17]. As for any varices, the pooled sensitivity and specificity were 0.78 and 0.67, respectively. As for clinically significant esophageal varices, the pooled sensitivity and specificity were 0.81 and 0.66, respectively. More recently, Ma et al. conducted a meta-analysis of 16 studies to compare the diagnostic accuracy of liver vs. spleen stiffness for the diagnosis of gastroesophageal varices [18]. The authors found that the sensitivity and specificity of liver stiffness for the diagnosis of gastroesophageal varices were 0.83 (95% confidence interval: 0.78–0.87) and 0.66 (95% confidence interval: 0.60–0.72), respectively; those of spleen stiffness were 0.88 (95% confidence interval: 0.83–0.92) and 0.78 (95% confidence interval: 0.73–0.83), respectively. Importantly, the spleen stiffness had a significantly higher diagnostic accuracy than the liver stiffness (summary receiver operating characteristic curve value: 0.88 vs. 0.81, $p < 0.01$; diagnostic odds ratio: 25.73 vs. 9.54, $p < 0.01$).

## 7. Capsule endoscopy for diagnosis of gastroesophageal varices

Until now, two meta-analyses were published regarding this topic. In 2014, a Cochrane review of 15 studies including 936 patients with liver cirrhosis analyzed the diagnostic performance of capsule endoscopy for the diagnosis of esophageal varices [19]. As for any varices, the pooled sensitivity and specificity were 0.848 and 0.843, respectively. As for large varices, the pooled sensitivity and specificity were 0.737 and 0.905, respectively. More recently, McCarty et al. systematically reviewed the data from 17 studies regarding wireless capsule endoscopy

for the diagnosis of esophageal varices [20]. As for any varices, the pooled sensitivity and specificity were 0.83 and 0.85, respectively. As for medium to large varices, the pooled sensitivity and specificity were 0.72 and 0.91, respectively.

## 8. Computed tomography scans for diagnosis of gastroesophageal varices

There are at least two meta-analyses regarding the value of computed tomography scans for the diagnosis of gastroesophageal varices. The first meta-analysis included 11 studies [21]. As for esophageal varices, the sensitivity and specificity were 0.896 and 0.723, respectively; as for gastric varices, the sensitivity and specificity were 0.955 and 0.658, respectively. The second meta-analysis included 17 studies [22]. As for any varices, the sensitivity and specificity were 0.87 and 0.80, respectively; as for any esophageal varices, the sensitivity and specificity were 0.87 and 0.81, respectively; as for any gastric varices, the sensitivity and specificity were 0.86 and 0.79, respectively. As for high-risk varices, the sensitivity and specificity were 0.87 and 0.88, respectively; as for high-risk esophageal varices, the sensitivity and specificity were 0.87 and 0.88, respectively; as for high-risk gastric varices, the sensitivity and specificity were 0.83 and 0.97, respectively. More recently, a retrospective study found that a diameter of esophageal varices of 3.9 mm on computed tomography scans might be the optimal cut-off value for the diagnosis of high-risk varices [23].

## 9. Endoscopic ultrasound

Researchers also explored the value of endoscopic ultrasound in the diagnostic evaluation of gastroesophageal varices [24]. Endoscopic ultrasound was inferior to conventional endoscopy in the diagnosis and grading of esophageal varices, but superior in the evaluation of para- or peri-esophageal veins and gastric varices. More importantly, the detection of para- or peri-esophageal veins by endoscopic ultrasound predicted the risk of bleeding and outcomes.

## 10. Conclusions

Alternative diagnostic tests of varices in liver cirrhosis have been widely explored in numerous studies. Several scores for the assessment of liver fibrosis are readily available, but have relatively low diagnostic accuracy. PSR and liver and spleen stiffness are noninvasive and have moderate diagnostic accuracy. By comparison, contrast-enhanced computed tomography and capsule endoscopy have relatively high diagnostic accuracy, but are expensive and potentially invasive (exposure to radiation). Thus, a diagnostic algorithm according to the cost and diagnostic performance of various diagnostic tests and clinical necessity should be

considered. In detail, PSR and liver and spleen stiffness should be the first step for the non-invasive diagnosis of varices; if a thorough evaluation of severity of liver diseases is simultaneously needed, contrast-enhanced computed tomography scans should be preferred and arranged earlier; if available, an endoscopic ultrasound can be performed to more accurately detect the para- or peri-esophageal veins.

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Chapter 4

# Correlation Between Transthoracic Contrast-Enhanced Ultrasound and Pulse Oximetry in Hepatopulmonary Syndrome Diagnosis

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**Abstract**

The prevalence of hepatopulmonary syndrome (HPS) in the setting of cirrhosis ranges between 4 and 47% and its presence increases the mortality rate, especially when hypoxemia is present. Our study aim was to fix whether there is a correlation of results between two simple and non-invasive procedures such as transthoracic contrast-enhanced ultrasound (CEUS) and pulse oximetry, used for early detection of HPS in patients with liver cirrhosis, having as endpoint the improvement in their outcome. The rapid lung enhancement and delayed left ventricle enhancement of the saline solution, after at least three systolic beats during CEUS and pulse oximetry showing a $SaO_2 < 95\%$, were correlated and considered positive for the diagnosis of HPS. One hundred and sixty-five (44%) of the total of 375 patients diagnosed with liver cirrhosis enrolled in the current study, with or without respiratory symptoms (dyspnea, clubbing, distal cyanosis, cough and/or spider angioma), showed positive criteria for HPS diagnosis during CEUS. $SaO_2 < 95\%$ and $PaO_2 < 70$ mmHg were found in 123 patients (33%) during pulse oximetry investigation. Pearson correlation index showed a good correlation between lung and heart CEUS findings and pulse oximetry ($r = 0.97$) for HPS diagnosis. CEUS and pulse oximetry results correlate and rapidly diagnose HPS, a highly fatal complication of liver cirrhosis (LC), guiding the future treatment by speeding up orthotopic liver transplant OLT recommendations to improve the survival rates.

**Keywords:** transthoracic contrast-enhanced ultrasonography, pulse oximetry, liver cirrhosis, hepatopulmonary syndrome, hypoxemia

## 1. Introduction

The hepatopulmonary syndrome (HPS) represents a complication of liver cirrhosis characterized by a gross dilatation of the pulmonary precapillary and capillary vessels, an increase in the number of dilated vessels, portopulmonary anastomoses, pleural and pulmonary arteriovenous shunts. It can be diagnosed when the triad represented by liver disease, impaired oxygenation and intrapulmonary vascular abnormalities, referred to as intrapulmonary vascular dilatations (IPVDs) coexist [1]. The prevalence of pulmonary complications associated with liver cirrhosis ranges between 4 and 47%, worsening the evolution and prognosis, especially when hypoxemia is present [2, 3]. According to the medical literature focused on the current topic, 23% of patients with HPS have an average survival rate around 24 months, compared to 63% of patients without HPS. Survival can be further worsened in case of comorbidities or advanced age [4]. Respiratory signs and symptoms are common in patients with liver cirrhosis, no matter the stage of the disease. Intrapulmonary vascular complications of liver cirrhosis consist of hepatopulmonary syndrome (HPS) and portopulmonary hypertension. HPS appears when intrapulmonary blood shunting impairs arterial gas exchange [5], and portopulmonary hypertension occurs when pulmonary arterial constriction leads to increased pulmonary arterial pressure [6]. The latter, although rare, can cause pulmonary complication, which worsens the morbidity and mortality in patients with liver dysfunction. The outcome of patients with advanced liver disease, complicated with pulmonary involvement, can be influenced even in the setting of orthotopic liver transplant, due to chronic hypoxemia installed during the evolution of cirrhosis influencing the prognosis. A key factor in the diagnosis of HPS is the exclusion of causes other than HPS that may be involved in cirrhosis and characterized by hypoxemia (cardiopulmonary abnormalities, pulmonary atelectasis, pneumonia, ascites, pulmonary edema or hepatic hydrothorax) [7]. The challenge for physicians working in the field of hepatology is to raise the idea of establishing new methods for a conventional, rapid and simple diagnosis of pulmonary involvement during the evolution of liver cirrhosis, in order to improve as much as possible the outcome of possible curative treatment.

HPS is defined by a widened alveolar-arterial oxygen gradient (age corrected) in room air, with or without hypoxemia. It results from intrapulmonary vascular dilatations in the presence of hepatic dysfunction and/or portal hypertension [8, 9].

The development of pulmonary vascular dilatation has as pathogenic mechanism a pulmonary overproduction of endogenous nitric oxide (NO) [10]. According to studies focused on the topic in the last two decades, a theory can be formulated according to which endothelin-1 and tumor necrosis factor-$\alpha$ may play a role in pulmonary microvascular tone modulation [11, 12]. The contributing factors to the process of pulmonary microvascular dilatation in HPS include angiogenesis, vascular remodeling, pulmonary arteriovenous shunts and portopulmonary venous anastomoses [13, 14].

Trough this pathogenic mechanism, the rapid or direct passage of mixed venous blood into the pulmonary veins is responsible for the pulmonary vascular dilatation. The mismatch of ventilation-perfusion sequence produces a deficit in the blood oxygenation. The inhibition of hypoxic vasoconstriction produces an increased blood flow and preserved alveolar ventilation.

The alveolar-arterial oxygen tension difference—≥15, or ≥20 mmHg for patients aged >64 is considered as a very sensitive index of early arterial deoxygenation in HPS, and this difference being overload before arterial oxygen tension becomes abnormally low [8]. On the other hand, the alveolar-capillary interface is too wide to allow for complete equilibration of carbon monoxide with hemoglobin, thus being translated in reducing the diffusing capacity of the lungs for carbon monoxide.

Patients complain of symptoms correlated not only with the subsequent liver disease, but also with the respiratory signs and symptoms, usually revealing dyspnea and cyanosis. The management of these patients requires the exclusion of other causes for such respiratory symptoms, because chronic obstructive pulmonary disease and pulmonary fibrosis can coexist in approximately 30% of patients with HPS [15]. Dyspnea ("platypnea") and hypoxemia ("orthodeoxia") are characteristically worsened in the upright position and improved by lying supine, resulting from a gravitational increase in blood flow through dilated vessels in the lung bases [16].

According to the pathogenic definition, the diagnosis of HPS requires evidence of pulmonary vascular dilatation and hypoxemia, with no cardiopulmonary disease history. To stage the severity of the disease, it is required to investigate the arterial blood gas tension at rest, while breathing room air and in the sitting position. A sensitive and non-invasive tool for the detection of pulmonary vascular dilatation is the contrast-enhanced transthoracic echocardiography after injection of hand-agitated normal saline. During the first pass, microbubbles are physiologically trapped and absorbed by alveoli, and they should not be seen in the left atrium. The passage of saline microbubbles through abnormally dilated lung vessels requires more than three cardiac cycles to reach left heart chambers [17]. In contrast, the immediate enhancement of saline microbubbles in the left atrium raises the suspicion of an intracardiac right-to-left shunt [18]. The alternative to CEUS investigation is scintigraphic perfusion scanning, which uses the technetium-99-labeled albumin macroaggregates >20 μm in diameter. The uptake of tc-99-labeled albumin macroaggregated in other organs occurs in case of right-to-left shunt, while the trapping of albumin macroaggregates in pulmonary circulation is characteristic for HPS [19].

The present management of HPS lacks of efficient therapy solutions, until the OLT is available. Starting from the pathogenic mechanism, physicians investigated several classes of drugs such as β-blockers, cyclo-oxygenase inhibitors, systemic corticosteroids, cyclophosphamide, inhaled NO, and NO inhibitors, but without a real benefit in oxygenation improvement or pulmonary vascular dilatation. The only efficient treatment in case of severe and refractory hypoxemia is the oxygen supplementation, with complete resolution in more than 80%, according to study results [8, 20]. The presence of HPS offers exception points in MELD scoring and an advantage for patients to occupy better places on waiting lists for OLT [21]. Without OLT, the prognosis for HPS is poor, with mortality around 41% of patients over a mean period of 2.5 years [22]. The literature data do not provide reliable clinical predictors or diagnosis guidelines for the outcome of HPS [23].

A retrospective cohort analysis of data submitted to the United Network for Organ Sharing studied the effects of room-air oxygenation of patients with HPS and the pre- and post-transplantation outcomes. Patients with HPS were given MELD exception points and prioritized

for liver transplantation due to their high pre- and post-transplantation mortality. Comparing the overall survival rates of patients with and without HPS, transplant recipients with more severe hypoxemia had increased risk of death after liver transplantation. The overall mortality was significantly lower among waitlist candidates with HPS (hazard ratio = 0.82; 95% CI: 0.70–0.96), having the OLT before the deterioration of tissue oxygenation and liver dysfunction, due to exception MELD points given, which provided an advantage for a rapid transplant [24].

The aim of our study was a possible correlation between contrast-enhanced ultrasound (CEUS) findings on heart and pulse oximetry, in order to early detect HPS, as a prognostic factor for orthotopic liver transplant (OLT) success [25].

## 2. Methods

Demographic data, etiology and severity scores were recorded. For the diagnosis of HPS, we used the classical triad: presence of chronic liver disease, an increased alveolar-arterial oxygen gradient, and evidence of right-to-left intrapulmonary shunt (IPS) [26]. In order to determine the HPS diagnosis, we used the classical charts provided by the guidelines for transplant candidates (**Table 1**). The diagnosis of liver cirrhosis was based on clinical, biochemical, ultrasound, and upper endoscopy criteria. The patients with liver cirrhosis were classified according to MELD scores, considering exception points according to international recommendations. The contrast-enhanced echocardiography (CEUS) [27], technetium-99m-labeled macroaggregated albumin (Tc-99m MAA) scanning [28], and pulmonary arteriography are the current imagistic tools to diagnose the IPS. We correlated transthoracic CEUS findings with pulse oxymetry as a screening test for detecting IPS in 375 patients diagnosed with liver cirrhosis between December 2009 and June 2016 in Gastroenterology Department of Clinical Emergency Hospital "St Apostle Andrew" of Constanta County.

| Criteria | Data requirements |
|---|---|
| Strict HPS criteria | Alveolar-arterial gradient ≥15 mmHg, or ≥20 mmHg if age older than 60 years |
| | Intrapulmonary shunting on transthoracic echocardiogram or >6% shunt fraction on macroaggregated albumin scan |
| | No evidence of severe restrictive or obstructive pulmonary disease |
| Hypoxia/hypoxemia+ IP shunts | Hypoxemia defined as:<br>• $PaO_2$ <70 mmHg on room air or<br>• Pulse oximetry ≤96% (room air or supplemental $O_2$) |
| | Intrapulmonary shunting (right → left bubbles on echocardiogram after three cardiac cycles and/or free text stating "intrapulmonary shunting") |
| | No evidence of concurrent cardiopulmonary disease |

**Table 1.** Inclusion criteria defining OLT waitlist candidates with HPS based on exception narrative data [28].

All patients were examined by chest X-ray and pulmonary function tests (to rule out common intrinsic pulmonary disorders such as chronic obstructive pulmonary disease). We used as a contrast agent of hand-agitated saline solution, in order to produce microbubbles with a mean diameter of up to 10 μm injected through a peripheral vein. Unlike blood, microbubbles resonate at a frequency similar to clinical transducer frequencies, which make ultrasounds to be reflected. Under normal circumstances, only the right heart chambers are opacified, and the microbubbles are trapped in the pulmonary capillaries (mean diameter, 8 μm). The presence of contrast in the left chamber suggests an arteriovenous connection. In patients with intracardiac shunts, a small amount of contrast is usually recorded in the left chambers within 1 or 2 cardiac cycles after its appearance in the right-side chambers (early shunt). On the contrary, late arrival of contrast in the left atrium after a time delay of 4–8 cardiac cycles is diagnostic for HPS (delayed shunt) and is done by the time required for passage through the pulmonary circulation [27]. Measurement of $SaO_2$ was performed with a portable pulse oximeter. In all patients, the measurements were performed at ambient $O_2$ partial pressure in supine position. We have chosen a $SaO_2$ value of <95% in order to detect all HPS patients with a $PaO_2 < 70$. The correlation of rapid lung enhancement and delayed left ventricle enhancement of the saline solution, after at least three systolic beats in the left ventricle during CEUS and pulse oximetry showing a $SaO_2 < 95\%$ was considered positive for the diagnosis of HPS [29].

## 3. Results

A total of 375 patients diagnosed with liver cirrhosis were enrolled in our study. The majority of patients were male (251/375). The average age was 66.04 years (SD 8.81). The etiology of liver cirrhosis was alcohol abuse in 39% (146/375) of patients, viral hepatitis B (VHB) in 28% (105/375) of patients, viral hepatitis C (VHC) in 21% (79/375) of patients, and the rest of 12% (45/375) having uncommon etiologies. Severity in MELD score divided our patients in three groups according to which we could fix the prognosis and the need of transplantation (**Table 2**).

According to present international recommendations, we decided upon exception points for those patients meeting the criteria for MELD exception: patients with $PaO_2 < 60$ mmHg on room air at rest in the sitting position, arterial blood gas result provided, patients with pulmonary vascular dilatation documented by a positive transthoracic contrast echocardiography, patients with absence of significant alternative pulmonary disease to explain severe hypoxemia (chest X-ray, pulmonary function tests, and chest computed tomography reports), patients with moderate or severe pulmonary function tests changes or significant chest X-ray abnormalities or MAA scan positive for intrapulmonary shunting) (**Table 3**). From the total of 375 patients studied, 165 (44%) presented respiratory symptoms. Pulse oximetry showed alterations, such as $SaO_2 < 95\%$ and $PaO_2 < 70$ mmHg in 123 patients (33%). From 375 patients diagnosed with LC, with or without present respiratory signs and/or symptoms (dyspnea, clubbing, distal cyanosis, cough and/or spider angioma) referred to CEUS examination, 105 (28%) had rapid lung enhancement and delayed left ventricle enhancement of the contrast agent (**Figures 1–3**). $PaO_2$ was less than 70 mmHg in all 105 HPS patients (100%) versus 12 (14.76%) of non-HPS patients ($P < 0.0001$). Pearson correlation index showed a good correlation between lung and heart CEUS findings and pulse oximetry ($r = 0.97$) in HPS diagnosis.

| Variable | HPS (no, %) | Non-HPS (no, %) |
|---|---|---|
| Mean age (IQR) | 66.04 ± 8.81 (95% CI, 58.44–74.85) | 63.10 ± 10.71 (95% CI, 61.55–64.65) |
| **Gender** | | |
| Males | 128 (50.99) | 123 (49.00) |
| Females | 59 (47.58) | 65 (52.41) |
| **Race** | | |
| Caucasians | 92 (87.61) | 243 (90) |
| Blacks | 2 (1.90) | 1 (0.37) |
| Asians | 11 (10.47) | 26 (09.62) |
| **Ethnicity** | | |
| Romanian | 51 (48.57) | 173 (64.07) |
| Turcs/tatars | 8 (7.61) | 19 (7.03) |
| Moldavians | 4 (3.80) | 9 (3.33) |
| Macedonians | 31 (29.52) | 42 (15.55) |
| Other | 11 (10.47) | 27 (10) |
| **Primary diagnosis** | | |
| HCV | 27 (25.71) | 52 (19.25) |
| HBV | 31 (29.52) | 74 (27.40) |
| Alcohol | 39 (37.14) | 106 (39.25) |
| HVD | 5 (4.76) | 18 (6.66) |
| Autoimmune | 2 (1.90) | 4 (1.48) |
| NASH/criptogenetic | 1 (0.95) | 9 (3.33) |
| Other rare causes | – | 5 (1.85) |
| **MELD score, median (IQR)** | 14 (11–22) | 16 (11–24) |
| **MELD score categories** | | |
| <15 | 47 (44.76) | 156 (57.77) |
| 15–20 | 34 (32.38) | 76 (28.14) |
| >20 | 14 (13.33) | 38 (14.07) |
| **MELD exceptions** | | |
| $PaO_2$ < 60 mmHG (22 pts) | 5 (4.76) | – |
| $PaO_2$ = 51–55 mmHG (24 pts) | 4 (3.80) | – |
| $PaO_2$ < 50 mmHG (26 pts) | 1 (00.95 | – |
| **History of ascites** | 84 (80.00) | 229 (84.81) |
| **History of liver decompensations** | 74 (70.47) | 172 (63.70) |

**Table 2.** Baseline clinical and demographic characteristics of HPS and non-HPS patients.

http://dx.doi.org/10.5772/intechopen.68550

| $PaO_2$ | Exception points for MELD scoring for HPS |
|---|---|
| 56–59 mmHg | 22 MELD points |
| 51–55 mmHg | 24 MELD points |
| <50 mmHg | 26 MELD points |

**Table 3.** Allocation of exception points for HPS in MELD scoring system [28].

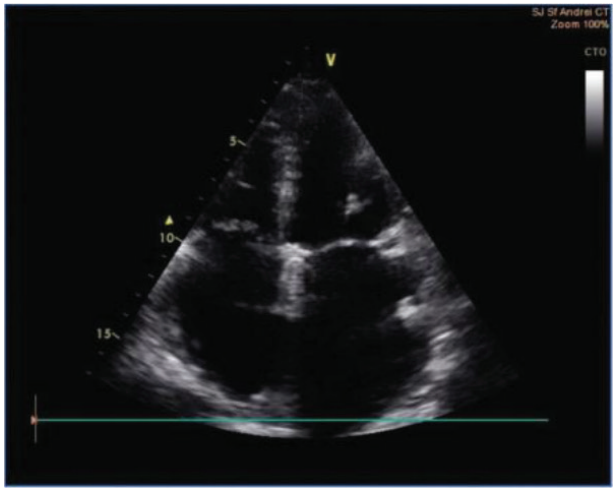


**Figure 1.** Contrast-enhanced echocardiogram. Apical four-chamber view before contrast injection.

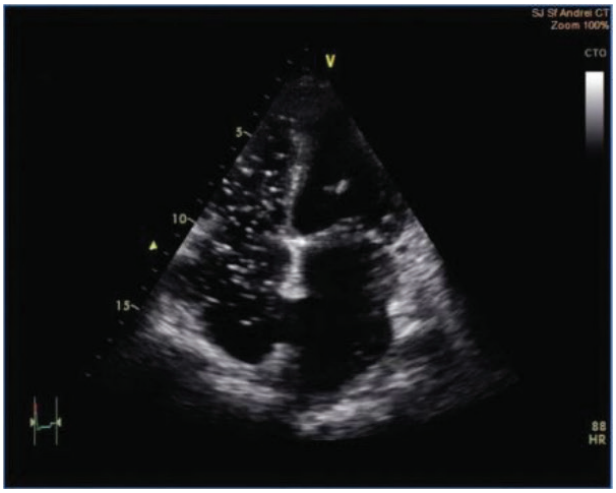


**Figure 2.** Contrast-enhanced echocardiogram. Apical four-chamber view after contrast injection (agitated saline) showing the presence of bubbles in the right chambers and no bubbles in the left chambers after the first sistola.

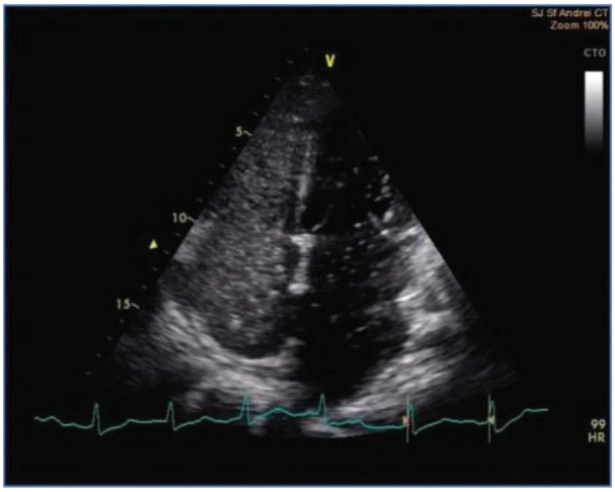


**Figure 3.** Contrast-enhanced echocardiogram. Apical four-chamber view after contrast injection (agitated saline) showing the presence of bubbles in the right heart chambers and the appearance of bubbles in the left heart chambers, late, after the forth sistola.

## 4. Discussion

HPS was defined as a triad of portal hypertension with or without hepatic dysfunction, intrapulmonary vascular dilatation or shunting, and hypoxemia [30]. Hypoxemia was defined by $PaO_2$ cutoff level of less than 70 mmHg in an arterial blood sample to pick up these patients for further evaluation by CEUS. This arterial $PO_2$ cutoff level was suggested by previous researchers [31], who found that patients with $PaO_2$ of more than 70 mmHg were unlikely to have HPS.

In the current study, among 375 patients diagnosed with liver cirrhosis, 105 patients (28%) met the clinical, laboratory and imagistic criteria of HPS. HPS shows a wide variability in prevalence in different studies, ranging from 4 to 47% among cirrhotic patients [1, 4, 32], depending on the diagnostic criteria and the cutoff levels used for hypoxia. In our study, $PaO_2$ was less than 70 mmHg in 100% of HPS patients versus 12% of non-HPS patients, in which pulmonary function tests were used to diagnose chronic intrinsic pulmonary disease. All patients with positive CEUS findings had arterial $PaO_2$<70 mmHg and were qualified for the diagnosis of HPS. CEUS was proved by previous investigators to be a useful sensitive and specific screening test for HPS even in early stages of liver dysfunction and even in whom the lung scintigraphy was still negative [33]. Some authors suggested transesophageal CEUS as a gold standard [34, 35]. However, others argued that transthoracic CEUS has the same accuracy as transesophageal CEUS in determining the presence of right to left shunt. Proper timing of left atrial opacification by microbubbles during the cardiac cycle was considered a distinguishing step in the transthoracic CEUS between intracardiac and intrapulmonary shunting [36]. Transesophageal CEUS might have higher sensitivity than transthoracic CEUS

because it allows the contrast to be seen when entering from the pulmonary veins [37, 38]. However, transthoracic CEUS is diagnostic in the majority of cases. In addition, esophageal varices are relatively common in these patients, and this can be considered as a relative contraindication in transesophageal CEUS performing [29, 39].

According to their correlated results, the transthoracic CEUS and pulse oximetry could be inserted in the algorithm of liver cirrhosis staging, in order to select those patients in need for a more rapid indication of OLT. Both methods provide data regarding the pulmonary dysfunction during liver cirrhosis evolution, improving the outcome after OLT, especially in HPS patients with moderate or severe hypoxemia. The presymptomatic stage of HPS can be correctly diagnosed using the combination of these two methods, making the algorithm of liver cirrhosis staging more accurate.

## 5. Conclusion

Our study showed a good correlation between lung and heart CEUS findings and pulse oximetry in HPS diagnosis. When correlated, these two simple, non-invasive, low-cost and rapid methods can easily diagnose HPS, a highly fatal complication of liver cirrhosis, which can worsen the outcome of patients even after OLT.

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Chapter 5

# Pulmonary Complications of Liver Cirrhosis: A Concise Review

Nwe Ni Than

Additional information is available at the end of the chapter

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**Abstract**

Pulmonary complications, in the form of hepatopulmonary syndrome (HPS), portopulmonary hypertension (PPH), and hepatic hydrothorax (HH), are rare occurrences in patients with portal hypertension and liver cirrhosis. These complications are associated with high morbidity and mortality. The only effective therapy is liver transplantation in patients who are suitable. In this chapter, each condition will be outlined in detail from clinical presentations to diagnosis and treatment as well as the challenges that clinicians may have encountered in managing patients with these complications.

**Keywords:** hepatopulmonary syndrome, portopulmonary hypertension, hepatic hydrothorax, liver transplantation

## 1. Introduction

Pulmonary complications in patients with chronic liver disease and portal hypertension include hepatopulmonary syndrome (HPS), portopulmonary complications (PPH), and hepatic hydrothorax (HH) (**Figure 1**). They are associated with increased morbidity and mortality and therefore, high suspicion of index is required to make earlier diagnosis and subsequently, to early treatment. The only effective treatment is liver transplantation (LT). All patients suitable for liver transplantation should be screened for potential pulmonary complications because earlier diagnosis gives better survival post liver transplantation. HPS is more common than PPH and HH, and the best chance of survival in these patients is LT. Among all the three conditions, HH carries the best prognosis.

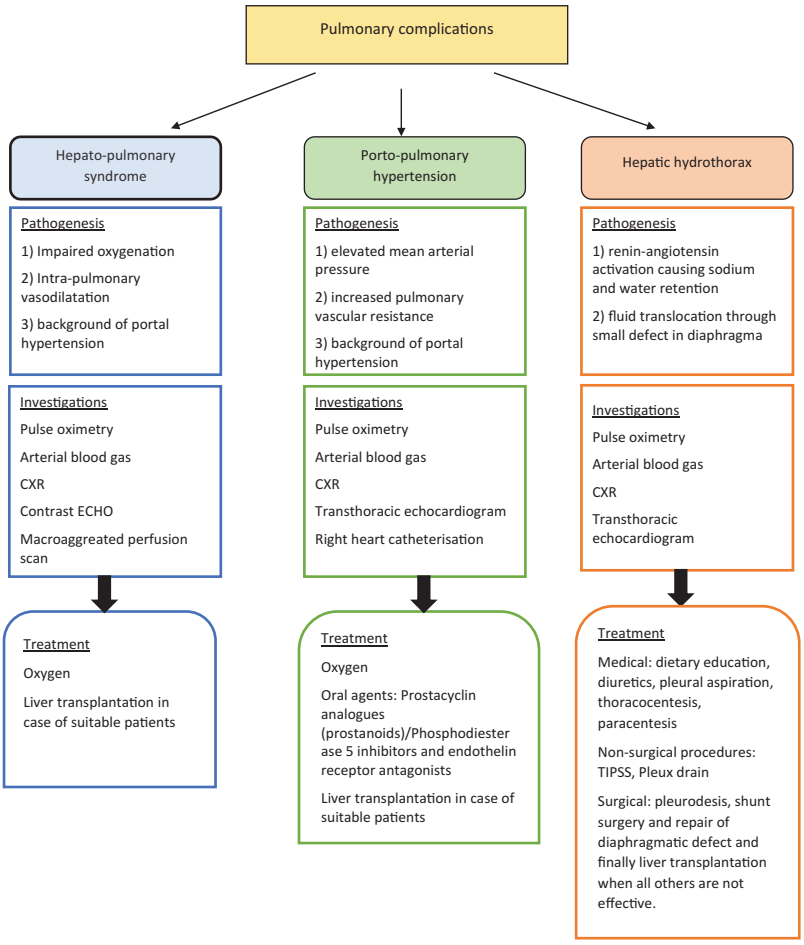


**Figure 1.** A step-wise approach to pulmonary complications of liver cirrhosis.

## 2. Hepatopulmonary syndrome

### 2.1. Background

Hepatopulmonary syndrome (HPS) is first described in 1977 by Kennedy and Knudson [1] and defined as a defect in arterial oxygenation caused by the presence of intrapulmonary vascular dilatation (IVPD) in the context of portal hypertension [2] (**Figure 2**). The estimated prevalence of HPS in liver cirrhosis is 4–32% [3]. In patients who were accessed for LT, the prevalence of HPS is approximately 10–30% [4]. HPS is usually diagnosed during the sixth decade of life and there is no specific association with gender or underlying cause of liver disease or model of end stage liver disease (MELD) [4, 5]. The established 5-year survival rate was 20% for HPS patients versus 32–63% for patients without HPS [5, 6].

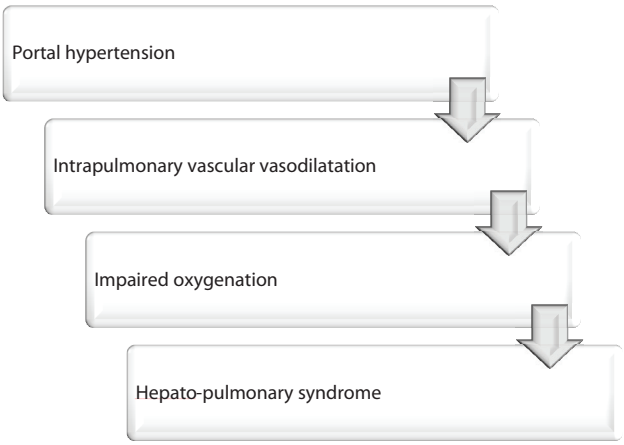


**Figure 2.** The sequence in development of HPS in liver cirrhosis.

### 2.2. Clinical features

Most patients with HPS present with dyspnea, orthopnea, platypnea, cyanosis, spider naevi, and finger clubbing [3, 7]. Platypnoea or orthodeoxia is defined as the presence of shortness of breath (dyspnoea) that worsens while sitting or standing and relieved by lying down. It is a common feature described in patients with HPS [7]. When patients with liver cirrhosis present with shortness of breath, the investigations should be done as early as feasible to avoid the delay in the diagnosis. Early diagnosis leads to reduction in patient's morbidity and mortality. The severity of HPS can be distinguished based on the level of hypoxemia as per the European Respiratory Task Force (**Table 1**) [8].

| Degree of severity | Level of hypoxaemia ($PaO_2$) |
|---|---|
| Mild | ≥80 mmHg |
| Moderate | ≥60–<80 mmHg |
| Severe | ≥50–<60 mmHg |
| Very severe | <50 mmHg |

**Table 1.** The severity of HPS as per level of hypoxaemia.

### 2.3. Pathogenesis

The pathogenesis of HPS is still unclear but the hallmark is thought to be due to intrapulmonary vasodilatation (IVPD), especially at the level of pre-capillary and capillary vasodilation [7]. IVPD is mediated by a number of endogenous vasoactive molecules, mainly endothelin-1 (ET-1) and nitric oxide (NO) [3, 9]. Portal hypertension increased the production

of vasoconstrictor ET-1, which stimulates the production of the ETB receptor at the level of the pulmonary microcirculation, with subsequent increase in eNOS activity causing vasodilatation [7, 9]. As a result of IVPD, nearly 20% or more of the cardiac output bypasses the functioning alveoli [2]. IVPD then causes arterial deoxygenation by three mechanisms: ventilation/perfusion mismatch, intrapulmonary shunting, and limitation of oxygen diffusion [7].

Angiogenesis is also considered to be an important phenomenon in the development of HPS [10] through upregulation of the vascular endothelial growth factor. Other mechanism suggested from experimental studies was vasodilation via increased carbon monoxide production through haem oxygenase [7]. The proposed pathogenesis of HPS was shown in **Figure 3**.

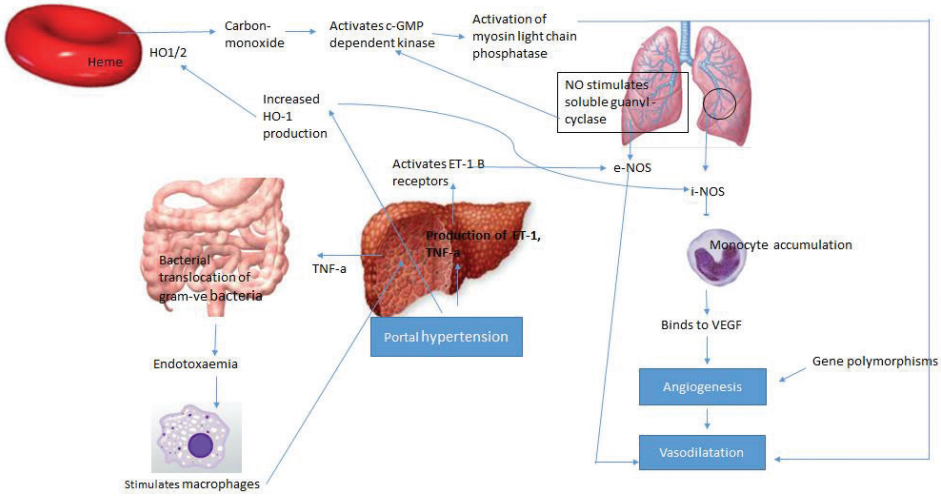


**Figure 3.** The pathogenesis of hepatopulmonary syndrome.

### 2.4. Investigations

In most centers, patients will usually undergo routine cardiopulmonary investigations during LT assessment. Bedside pulse oximetry is the first line screening investigation and oxygen saturation of less than 96%, has a sensitivity of 100% and specificity of 88% to detect $PaO_2 < 70$ mmHg [7, 11]. Arterial blood gas (ABG) sampling is required for the diagnosis of HPS to calculate the Alveolar-arterial (A-a) gradient [7]. PA-$aO_2$ gradient is the most important marker in diagnosing early stage of HPS [7] and the European Respiratory Society Task Force recommends a PA-$aO_2 \geq$ 15 mmHg for the diagnosis of HPS and the level of $PaO_2$ will determine the severity of the HPS [10] (**Table 1**). In suspected patients with HPS, ABG was performed on room air with patient sitting down first and the procedure is repeated 15 to 20 minutes in the standing up position. Orthodeoxia, which manifests as a decrease in $PaO_2$ of ≥4 mmHg or ≥5% from the supine to the upright position [12], and the increase in $PaO_2$ while breathing 100% oxygen, which should reach above 300 mmHg [7]. Orthodeoxia is a consequence of the increased V/Q mismatch and decreased cardiac output following the change from the supine to the upright position [7].

Chest radiography shows prominent pulmonary vascular markings in bilateral lower lobes, but finding is not specific for HPS [2]. Pulmonary function test should be performed to rule out other associated intrinsic pulmonary disorders. Contrast-enhanced echocardiography is the most sensitive test to demonstrate intrapulmonary shunting disease [2]. It is done using intravenous injections of agitated saline or indocyanine green to produce bubbles of at least 15 microns in diameter [2]. Normally, these microbubbles are trapped in the pulmonary vasculature and absorbed, but in intracardiac right to left shunts, these microbubbles are seen in the left heart within the first three cardiac cycles [7]. In HPS, the bubbles are seen in the left heart after the third heartbeat, usually between the third and sixth heartbeat due to intra-pulmonary shunting [2]. Studies have shown that transesophageal echocardiography is more sensitive than transthoracic echocardiography in demonstrating intrapulmonary shunting [7].

99 m Technetium-macroaggregated albumin (Tc-99 m MAA) lung perfusion scan is used widely in the diagnosis of HPS (**Figure 4**). Albumin macroaggregates with more than 20 μm in diameter are normally entrapped in the pulmonary vasculature [2]. In patients with intrapulmonary shunts, these albumin macroaggregates escape from the pulmonary vasculature and are taken up by other organs [2]. Normally, less than 5% of isotope reaches brain circulation compared to the lung, but in HPS patients, the fraction is more than 6% [7]. The major disadvantage of Tc-99 m MAA scan is its inability to differentiate intra-cardiac from intrapulmonary shunting. Pulmonary angiography is invasive, and hence, it is only reserved for those who did not have response to 100% oxygen therapy [7]. The baseline investigations and the findings found in HPS are illustrated in **Table 2**.

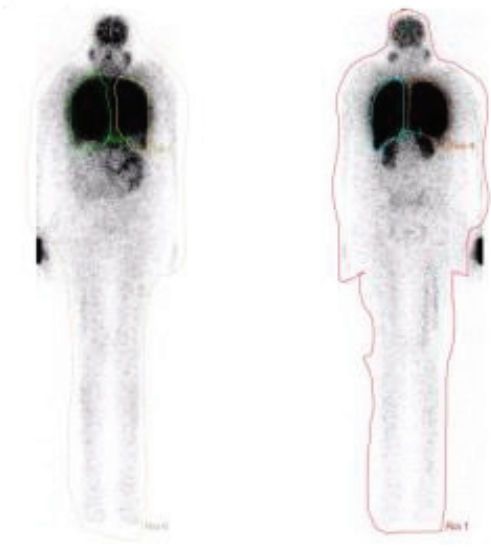

**Figure 4.** Whole body (Tc-99 m MAA) scan showed an increased uptake in within the lungs and thyroid with well visualization in the brain, kidneys, and liver.

| Screening methods | Findings |
|---|---|
| Pulse oximetry | Oxygen saturation <96% |
| Chest radiograph | Increased vascular markings |
| Lung function tests | Normal or reduction FVC or FEV1<br>Reduction in diffusing capacity of the lungs for carbon monoxide (DLCO-co) |
| Diagnostic tests | Findings |
| Arterial blood gas analysis | $AaO_2 \geq 15$ mmHg or<br>$AaO_2 \geq 20$ mmHg (in patients above 64 years of age) |
| Contrast echocardiography | Bubbles in the left cavities between the fourth and sixth beat |
| 99 m Tc-MAA | Cerebral uptake ≥6% |

**Table 2.** Screening and investigative methods used in HPS.

## 2.5. Treatment

### *2.5.1. Medical treatment*

Patients who experience severe dyspnea at rest and evidence of hypoxemia clinically should receive oxygen therapy [10]. Many studies have looked into treatment of HPS with nitric oxide inhalation, low consumption of L-arginine using methylene blue, aspirin, antibiotic usage to reduce intestine's bacterial translocation, somatostatin, indomethacin, garlic, and transjugular intrahepatic portosystemic shunt (TIPS), but none of them have not shown any particular benefit as long-term treatment of HPS [7].

Recent pilot randomized controlled study with norfloxacin did not show any improvement in gas exchange of HPS patient [13]. Initial studies suggested that garlic may have a role in the treatment of HPS by altering nitric oxide production [7]. A recent randomized controlled trial showed garlic supplementation, which was associated with a 24.66% increase in baseline arterial oxygen levels and 28.35% decrease in alveolar-arterial oxygen gradient [14]. It also shown that garlic supplementation may be beneficial in patients with HPS for the reversal of intrapulmonary shunts as well as for reducing hypoxemia and mortality, although study with higher number of patients are required to show clinical effectiveness [14].

One of the factors involved in the pathogenesis of HPS was tumor necrosis factor-alpha (TNF-a) and overproduction of TNF-a cause vasodilatation [4]. Hence, treatment with pentoxifylline (an inhibitor of TNF-a) although in recent pilot study [15] showed that pentoxifylline did not improve arterial oxygenation in advanced HPS, and tolerance was limited by gastrointestinal toxicity.

Enhanced pulmonary production of nitric oxide (NO) has been implicated in the pathogenesis of HPS, and NO inhibition with N(G)-nitro-L-arginine methyl ester (L-NAME) in both animals and humans with HPS has improved arterial hypoxemia [16]. A study [16] investigating the effect of nebulized L-NAME in patients with HPS showed that

the treatment decreased exhaled NO, mixed venous nitrite/nitrate, and cardiac output although systematic and pulmonary vascular resistance were increased. In contrast, ventilation-perfusion mismatching, intrapulmonary shunt, and, in turn, arterial deoxygenation remained unchanged [16].

*2.5.2. Transjugular intrahepatic portosystemic shunt (TIPSS)*

Recent systematic reviews of 10 studies with 12 patients showed that TIPSS is technically feasible to perform in patients with HPS, but overall benefit is unclear [17]. The current management did not advise TIPSS in patients with HPS.

*2.5.3. Liver transplantation (LT)*

The only effective treatment available for HPS is liver transplantation (LT), although LT is invasive and carries a high risk. Hence, patients should be accessed thoroughly prior to consideration of LT. After LT, 85% patients had significant improvement in gas exchange, although it can take up to 1 year for the abnormalities to normalize [2]. The mortality is higher for patient with HPS who underwent LT than those without HPS and the mortality is higher for those with marked hypoxemia ($PaO_2$ < 50 mmHg) and intrapulmonary shunting (shunt fraction > 20%) [2]. The established 5-year survival rate was 23% for HPS patients and 67% for patients without HPS [18]. For patients with HPS who are on LT waiting list should be monitored closely to prevent worsening of the conditions. The most challenging post LT is severe hypoxemia post-operative period with prolonged respiratory weaning that often resulted in death. Ten-year survival after LT in HPS patients stands at 64% [10] and post LT mortality rates obtained in these studies range between 7.7 and 33% [10].

Recent study showed that patients with HPS presented higher cardiac output, lower systemic vascular resistance, and higher progesterone and estradiol levels than patients without HPS [19]. The study showed that LT produced normalization of intrapulmonary vasodilatation in all patients as well as hyperdynamic circulation and hence, is a useful therapeutic option in patients with HPS [19]. Normalization of sex hormone levels after LT suggests that they could play a pathogenic role in the development of HPS [19].

*2.5.4. Other treatment options*

One of the recent management options for life-threatening hypoxemia in HPS patients is extracorporeal membrane oxygenation (ECMO) [20]. Monsel et al. reported the use of ECMO in preparation of LT in patients with refractory hypoxemia caused by a combination of acute respiratory distress syndrome (ARDS) and HPS [21]. The preliminary data showed that ECMO allowed the performance of successful LT by controlling gas exchange [3]. Auzinger et al. also reported the successful case of using ECMO for severe refractory hypoxemia after LT in HPS patients [20]. It could facilitate early ventilator weaning, thus prevented the need for the prolonged use of sedation and reduced complication associated with interventions [20]. However, the effectiveness of ECMO still has to be proven by future randomized trials.

## 3. Portopulmonary hypertension

### 3.1. Background

Portopulmonary syndrome (PPH) was first described in 1951 by Mantz and Craige [22]. PPH is characterized by the presence of elevated mean pulmonary hypertension in patients with portal hypertension due to increased pulmonary vascular resistance [4]. It is found in 2–10% of patients with cirrhosis [2] and reported among 5–8% of the patients with CLD who have undergone liver transplantation [23].

A recent retrospective review conducted in treatment-naïve patients with PPH within the United Kingdom national registry showed that patients with PPH had survival rates of 85, 60, and 35% at 1, 3, and 5 years [24]. The study mentioned that the prevalence of PPH was found to be 0.85 cases per 1 million and the mean age of diagnosis was 53 years [24]. Alcohol and hepatitis C were found to be the most common causes of PPH [24].

PPH results from arterial vasoconstriction linked to remodeling of the vasculature of the lung caused by prolonged portal hypertension and subsequently lead to pulmonary arterial hypertension (PAH) [9]. The condition is more common in females and in patients with autoimmune hepatitis [7, 25]. PPH can occur at any age but more common in fourth or fifth decade of life [4]. PPH occurs 4–7 years after patients are diagnosed with portal hypertension [26]. The severity of liver disease does not correlate with the severity of PPH. Without treatment, estimated 1-year survival in PPH is around 60% [23, 27].

### 3.2. Clinical features

Most patients are asymptomatic but clinical features of liver disease will be apparent. Patients usually present with features of right-sided heart failure such as dyspnea, orthopnea, chest pain, fatigue, and syncope [9]. On clinical examination, patient may present with tricuspid regurgitation murmur, loud pulmonary (P2) sound, diastolic murmur of pulmonary regurgitation, and features of right-sided heart failure evident by the presence of elevated jugular venous pressure, pulsatile liver, peripheral edema, and ascites [9]. The severity of PPH is classified based on degree of MPAP values: mild (25–35 mmHg), moderate (35–50 mmHg), and severe (>50 mmHg) [9].

The European Cardiologic Society and the European Respiratory Society Task Force have defined the diagnostic criteria for PPH as follow in **Table 3** [28]. According to the World Health Organization classification, PPH is located within PAH group 1 [29].

| **Diagnostic criteria for PPH** |
|---|
| Mean pulmonary arterial pressure (mPAP) >25 mmHg |
| Pulmonary vascular resistance (PVR) >240 dyn s $cm^{-5}$ |
| Pulmonary capillary wedge pressure <15 mmHg |

**Table 3.** Diagnostic criteria for portopulmonary hypertension (PPH).

### 3.3. Pathogenesis

The exact pathophysiology behind PPH is poorly understood but histologically, it is thought to be similar to the pathogenesis of idiopathic pulmonary arterial hypertension (PAH) [29]. Hyperdynamic circulatory state and high cardiac output are the hallmarks in most of the patients with PPH leading to increased shear stress on the pulmonary circulation [29]. Due to vascular shear stress, vasoactive, proliferative, and angiogenic mediators (including endothelin 1 (ET-1), vasoactive intestinal peptide, serotonin, thromboxane A2, interleukin 1, glucagon, and secretin) were released which lead to arterial changes seen in PPH [2, 4, 23, 27]. The main pathological abnormalities include proliferate arteriopathy, obliteration of the vascular lumen by endothelial and smooth muscle cells, formation of plexiform lesions, necrotizing arteritis, fibrinoid necrosis, and *in-situ* thrombi [23, 27]. Due to portosystemic shunts, bacterial endotoxins were found in pulmonary circulation from gastrointestinal tract and the recruitment of interstitial macrophages to clear those endotoxins also contribute to the development of PPH [30].

Genetic polymorphisms may play a role in the development of PPH. Finally, vasodilating mediators, such as nitric oxide (NO) and prostaglandin $I_2$ (prostacyclin), may be decreased in PPH [29]. Prostacyclin synthase, the enzyme responsible for prostacyclin synthesis, has been demonstrated to be deficient in the pulmonary endothelium of patients with PPH [4]. The illustration pathogenesis of PPH is shown in **Figure 5**.

### 3.4. Investigations

Since patient can be asymptomatic, high suspicion is required to diagnose this condition earlier, which can lead to earlier treatment and better prognosis. All baseline investigations such as ECG, CXR, blood gas analysis, and lung function tests have poor prognostic yield and did not reflect severity of PPH. In patient with PPH, CXR might show a prominent main pulmonary artery, cardiomegaly due to enlarged right cardiac chambers, and increased vascularity in the upper lobes [2, 4, 9]. Pulmonary function tests in patients with PPH would show decreased lung diffusion capacity and reduced lung volume [2, 4]. In arterial blood gas analyses, hypoxemia and hypocapnia associated with an elevated alveolar-arterial oxygen gradient would be seen [2].

Transthoracic echocardiogram showed right ventricular hypertrophy and right atrium dilatation, which is not usually specific to PPH [23, 27]. Transthoracic echocardiogram (TTE) is the screening tool used initially and it can identify patients with elevated pulmonary arterial systolic pressure (PASP). In those patients with elevated PASP, the next investigation is right heart catheter which can confirm the diagnosis of PPH. Usually, RV systolic pressure <30 mmHg was used to exclude PPH and if it is >50, patient is highly likely to have PPH [23]. Cardiac output (CO), mean pulmonary arterial pressure (mPAP), mean pulmonary arterial occlusion pressure (mPAOP), and pulmonary vascular resistance (PVR) can help to determine the nature and severity of the PPH [2, 27]. There are three main causes of elevated mPAP in liver disease patients and those are cirrhotic cardiomyopathy due to left ventricular dysfunction, the typical high-output state of cirrhosis, and PPH [27]. **Table 4** illustrates the difference findings noted in each condition.

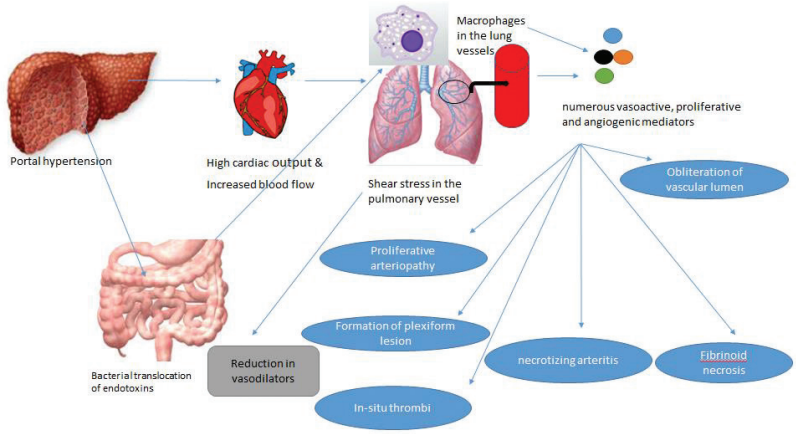


**Figure 5.** The pathogenesis of portopulmonary hypertension.

| | Cardiac output | mPAP | mPAPOP | PVR |
|---|---|---|---|---|
| Hyperdynamic state | Elevated | Elevated | Normal | Decreased |
| LV dysfunction | Low | Elevated | Elevated | Elevated |
| PPH | Low | Elevated | Low | Elevated |

**Table 4.** The difference findings for each conditions.

The severity of PPH and the progression of disease during the course of disease in patients with portal hypertension can only be investigated through invasive right heart catheterization. Hence, it will be useful to develop a sensitive biomarker which can detect disease presence, predict the severity, and treatment response. A recent prospective multicentre case-control study which studied the plasma level of macrophage migration inhibitory factor (MIF) in PPH patients seemed to show promising results [31]. It showed that MIF was higher in both the systemic and pulmonary circulations of patients with PPH compared with controls and correlated with hemodynamic indices of disease severity [31]. High levels of MIF were associated with an increased risk of death and MIF production may play a role in disease pathogenesis of PPH [31]. MIF can be an ideal novel biomarker in detecting disease presence and severity [31].

### 3.5. Treatment

Treatment strategies for PPH are derived from studies of idiopathic PAH and the aim of therapy is to provide symptomatic relief, to improve the quality of life and exercise capacity, and to facilitate liver transplant [23]. The only effective treatment in patients with PPH is liver transplantation in patients who are suitable after careful assessment. Medical therapies that have

been tried for PPH include endothelin receptor antagonists, phosphodiesterase 5 inhibitors, and prostacyclin analogs [2, 23, 27]. There are limited data evaluating the long-term survival of patients with PPH managed with medical therapy alone. Recent study from UK showed that phosphodiesterase 5 inhibitors were the most frequently used targeted therapy (63%) followed by prostacyclin analogs (12.7%), and endothelin receptor antagonists (10%) [32].

#### *3.5.1. General medical treatment*

In patient with significant hypoxemia, oxygen therapy is needed for improvement of symptoms. For those with significant edema and ascites, diuretics should be initiated. In patients with PPH, they are at risk of thrombosis and hence anticoagulation is recommended. However, in patients with liver cirrhosis had increased risk of variceal bleeding due to underlying portal hypertension and clinical judgment is required prior to starting anticoagulation in these group of patients.

Calcium channel blockers can be used due to their acute vasoreactive properties in PAH but can be dangerous in patients with PPH since it can result in worsening of portal hypertension because of their mesenteric dilatation properties [2, 23, 27]. TIPSS are not recommended in PPH since it can deteriorate PPH because of acute increase in preload causing increased cardiac output and mPAP, and then leads to worsening right ventricular strain and dysfunction [29].

#### *3.5.2. Specific therapies for PPH*

The therapies specific for PPH targeted to improve pulmonary vasoconstriction and vascular remodeling by altering three pathways: Prostacyclin analogs (prostanoids), phosphodiesterase 5 inhibitors, and endothelin receptor antagonists [2, 9, 27, 33]. Pulmonary vasodilators treatment should be employed with the aim of lowering mPAP < 35 mmHg, to minimize the risk of graft failure and to improve the overall outcome [42].

#### *3.5.3. Prostacyclin derivatives*

They are potent pulmonary as well as systemic vasodilators, and have antiplatelet aggregating and antiproliferative effects [27]. The most commonly used prostacyclin is epoprostenol and it is the only treatment that has been shown to improve survival in idiopathic PAH [27].

#### *3.5.4. Endothelin receptor antagonists*

Bosentan is an oral dual effective, nonselective receptor antagonist that blocks both endothelin A and B receptors [27], and it has been shown to be effective in the treatment of PPH showing clinical, functional, and hemodynamic benefits without significant hepatotoxicity in some small retrospective case series [29]. Bosentan is probably the therapy of choice for patients with PPH as it potentially improves pulmonary as well as portal hypertension [29]. It is potentially hepatotoxic and may cause deterioration in liver enzymes in about 10% of patients, and hence, close monitoring is needed [29]. A recent study showed that Child-Pugh B cirrhosis with PPH had significantly larger hemodynamic improvement with bosentan treatment [34].

It was also found that plasma concentrations of bosentan were higher in patients with child B cirrhosis than those observed in idiopathic PAH [34].

*3.5.5. Phosphodiesterase 5 inhibitors*

Phosphodiesterase-5 inhibitor therapy is efficacious in other causes of WHO group I pulmonary arterial hypertension [32]. They inhibit the growth of pulmonary vascular smooth muscle cells and lower mean pulmonary artery pressure and pulmonary vascular resistance by mediating vasodilation through guanosine monophosphate [2, 27]. Sildenafil is commonly used in PPH and reported to be effective in reducing mPAP and PVR [29]. Sildenafil is approved in a dose of 20 mg three times a day for treatment of PPH [35], and it should be considered as a bridging therapy before liver transplant for patients with PPH to delay the progression of the disease.

A recent single center retrospective study showed that sildenafil therapy resulted in improvement of WHO functional class with significant decrease in PVR, mPAP, and increase in cardiac output but no change in 6-min walk test over the period of 6 months treatment [32]. A recent retrospective study of all patients with PPH treated by oral pulmonary vasoactive drugs (PVD) (bosentan, ambrisentan, sildenafil, tadalafil) showed that oral PVD improved MPAP, PVR, and 6-min walk distance [36]. The study showed that oral PVD are safe, better tolerated in patients with cirrhosis, and did not showed any worsening of cirrhosis and these treatments improved hemodynamic conditions allowing patients access to liver transplantation eligibility [36].

*3.5.6. Liver transplantation*

LT is the definitive therapy for patient with PPH when medical therapy fails. LT should be considered in patients with mean pulmonary artery pressure (MPAP) <35 mmHg or MPAP between 35 and 50 mmHg with pulmonary vascular resistance (PVR) <250 dyn s $cm^{-5}$ [23, 37]. PPH is diagnosed in 2–6% of liver transplantation (LT) candidates [38]. Without LT, the survival rate for patients with PPH was found to be 38% at 3 years and 28% at 5 years [37]. Due to the severity of the condition and high mortality associated with it, patient with PPH should be assessed careful before considering LT. Perioperative mortality in patients with mean PAP >35 mmHg is significantly higher compared to those with mPAP < 35 mmHg [4, 23]. The outcome is worse in patients with moderate to severe PPH [mean pulmonary artery pressure (MPAP) ≥ 35 mm Hg] and associated with a perioperative mortality rate of 50% [37, 38].

Therefore, patient should be treated with medical therapy while awaiting LT to delay the progression of disease as well as to improve perioperative risk. The goal of therapy in patients with PPH, who are candidates for liver transplants, is to reduce mPAP <35 mmHg and the PVR < 400 dyn s $cm^{-5}$ before proceeding to liver transplant [29].

Patients on liver transplant waiting list are prioritized based on the model of end-stage liver disease (MELD) score but in patients with PPH, potentially important factors such as severity of PPH is not included which may affect survival. Recent retrospective cohort study of patients in the Organ Procurement Transplantation Network (OPTN) database with hemodynamics consistent with PPH [defined as mean pulmonary arterial pressure (mPAP) >25 mmHg and

pulmonary vascular resistance (PVR) ≥ 240 dynes.sec.cm-5 who were approved for a PPH-MELD exception between 2006 and 2014 showed that initial native MELD score and initial PVR were the only significant univariate predictors of waitlist mortality and remained significant predictors in a multivariate model [39]. The study showed that PVR and mPAP were not significant predictors of post-transplant mortality [39].

According to the European Respiratory Society Task Force, patients with mean pulmonary artery pressure < 35 mmHg can undergo a liver transplant, patients with mean pulmonary artery pressure of 35–45 mmHg should receive vasodilator therapy before transplant, and patients with mean pulmonary artery pressure > 45 mmHg should receive vasodilator therapy only [4, 7].

## 4. Hepatic hydrothorax

### 4.1. Background

Hepatic hydrothorax (HH) is a more common clinical entity compared to HPS and PPH and carries the best prognosis [9]. HH accounts for 2–3% of total pleural effusions [40]. However, in patients with portal hypertension, HH occurred in 5–10% of cases [41].

HH is caused by the accumulation of transudative effusion in patients who did not have underlying cardiopulmonary disease [42]. Majority of HH was noted on right side in 79.5% of cases followed by left sided and bilateral in 17.5 and 3%, respectively [40].

Since the pleural space is relatively small compared to the abdominal cavity with low compliance of the thoracic cavity, patients can become symptomatic with as little as 500 ml accumulation of fluid [42]. Like ascites, HH can become spontaneously infected, a condition known as spontaneous bacterial empyema (SBEM), which carries a mortality of up to 20% [42]. The incidence of SBEM was noted to be 13% in a prospective study [43] and interestingly, up to 40% of SBEM patients are not associated with incidence of spontaneous bacterial peritonitis (SBP) [43].

### 4.2. Clinical features

The clinical presentation is usually found in patients with cirrhosis and portal hypertension, i.e., ascites, spider naevi, asterixis, hepatosplenomegaly, and caput medusa. Patients with HH can present with pulmonary symptoms as in shortness of breath, cough, hypoxemia, or respiratory failure associated with large pleural effusions [40]. SBEM should always be suspected when patients develop fever, pleuritic chest pain, or features of liver decompensation.

### 4.3. Pathogenesis

The pathogenesis of HH is similar to those leading to ascites in portal hypertension [40, 41]. Portal hypertension and splanchnic vasodilatation are the main pathways leading to fluid accumulation as a result of decrease in effective blood volume which then activate renin-angiotensin system leading to sodium and water retention [9]. Particularly in HH, it is thought to be a

consequence of ascitic fluid translocation through congenital diaphragmatic defects into the pleural cavity [42]. These defects, normally covered with pleuroperitoneum, were most frequently seen in the right hemi-diaphragm and usually smaller than 1 cm in size [42]. Ascites accumulation increases the intraperitoneal pressure which causes rupture of the pleuroperitoneal membrane and as a result, ascitic fluid can move into the low pressure pleural space [42]. This explanation for the appearance of hepatic hydrothorax is supported by studies showing intraperitoneal-injected radiotracer activity in the pleural fluid of such patients [44]. HH can happen due to hypoalbuminemia resulting in decreased colloid osmotic pressure [45] and lymphatic leakage from the thoracic duct [46].

### 4.4. Investigations

Patients with portal hypertension with pulmonary clinical features should be investigated thoroughly to rule out other causes of pulmonary and cardiac disorders. HPS and PPH should be investigated as part of differential diagnosis. The presence of pleural effusions is usually detected by thorough respiratory examination with findings of dullness to percussion, mediastinal shift, diminished or inaudible breath sounds, and pleural friction rub. In clinically suspected patients, pleural effusions can be confirmed with one of the imaging modalities such as chest X-ray (**Figure 6**), ultrasound scan, or CT chest. Echocardiogram should be performed to rule out underlying cardiac causes of effusions.

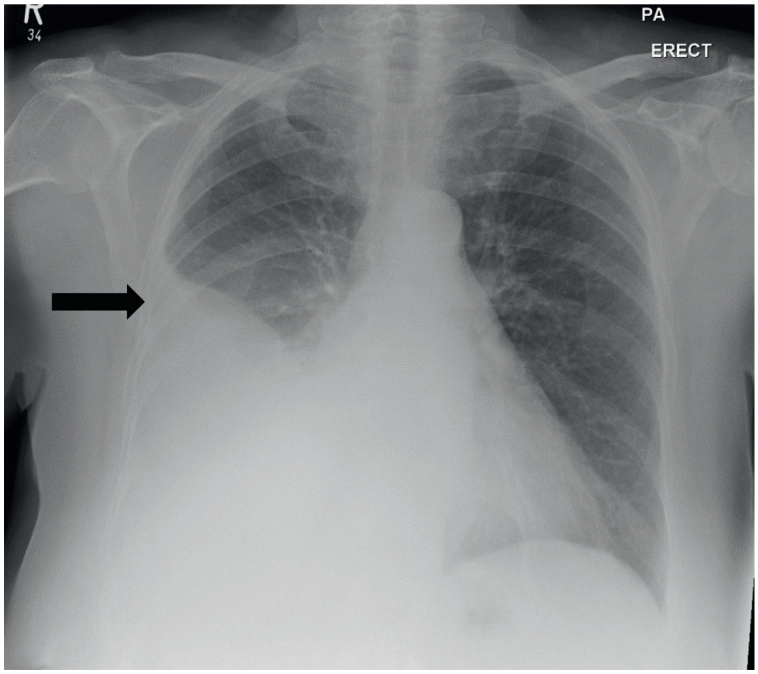


**Figure 6.** Chest X-ray showed the presence of right-sided pleural effusion.

Pleural fluid should be examined to rule out other causes leading to pleural fluid such as infection, inflammation, and malignancy. Pleural fluid should be aspirated using ultrasound and the sample should be sent for cell count, gram stain, culture, cytology, pH, total protein, albumin, lactate dehydrogenase (LDH), and amylase. Diagnosis of transudate is based on Light's criteria, which is shown in **Table 5** [47], since HH is transudate in nature.

| |
|---|
| *Light's criteria* |
| Pleural fluid total protein/serum total protein ratio <0.5 |
| Pleural fluid LDH/serum LDH <0.6 |
| Pleural fluid LDH < two thirds of the upper limit of normal serum LDH |
| *Other investigative parameters* |
| Total protein <2.5 g/dl |
| Pleural fluid lactic dehydrogenase (LDH) <200 IU |
| Serum pleural to fluid albumin gradient >1.1 g/dl |
| Glucose level similar to that of serum |
| pH 7.4–7.55 |
| Polymorphonuclear count <250 cells/$mm^3$ |

**Table 5.** Characteristics of pleural fluid in HH.

In patients with SBEM, pleural fluid has high Polymorphonuclear cell counts >250 cells/$mm^3$ with positive culture or >500 cells/$mm^3$ in patients with negative culture without any evidence of underlying chest infection/pneumonia or exudative features of infection [40].

### 4.5. Treatment

#### *4.5.1. Medical therapy*

The role of medical therapies is to relieve symptoms and prevent the complications of HH in patients awaiting liver transplantation or to palliate symptoms in those who are not transplant candidate [42]. Treatment is similar to the treatment of ascites which include dietary salt restriction, diuretic therapy, and drainage of fluid either from abdomen or pleural space.

The management of dietary sodium is important to prevent re-accumulation of fluids and dietary education should be given to patients. Diuretic therapies with furosemide 40–80 mg once daily with or without addition of spironolactone 50–400 mg OD are used in patients who are tolerant of diuretic therapy. Urinary sodium should be checked before and during therapy to adjust diuretic dosage as per clinical response. In patients with refractory ascites, the other treatment modalities can be used. These include paracentesis, thoracentesis, insertion of chest drain tube, indwelling tunneled pleural catheter (PleurX) insertion, insertion of transjugular intrahepatic portosystemic shunt (TIPSS), pleurodesis, shunt surgery, and repair

of diaphragmatic defect [40, 41, 48–51]. Each treatment has its own advantages and disadvantages and should be selected as per patient's clinical condition.

In patients with HH and large volume ascites, ascites should be drained before draining pleural fluid to prevent the rapid accumulation of fluid in the pleural space after thoracentesis due to decreased intrathoracic pressure [40]. Thoracentesis is used for large pleural effusion in patient with significant dyspnea. Pleural fluid should be drained not more than 2 L of fluid at any time point to prevent expansion pulmonary edema. If patients required regular thoracentesis, they should be considered for therapies that provide long term symptom relief. Indwelling tunneled pleural catheter (PleurX) insertion is usually considered for patients in palliative setting.

TIPSS is effective in controlling ascites and hepatic hydrothorax, although the procedure did not improve the prognosis of patients with end-stage liver cirrhosis [40, 51]. TIPSS should be considered in patients with compensated liver cirrhosis and the factors associated with increased mortality in patients who had TIPSS are age >60 years, Child Pugh class C, presence of pre-TIPSS high model for end-stage liver disease (MELD) score >15 and high pre-TIPS creatinine levels >2 mg/dl [51]. Patients whose had high risk features described above should be considered for LT.

In patients with SBEM, the management is to treat underlying infection with broad spectrum antibiotics with or without inserting large bore chest drain tube.

#### *4.5.2. Liver transplantation*

In patients with refractory ascites who are Child Pugh C cirrhosis, LT should be considered first prior to other therapies. The presence of HH does not lead to more post-operative complications, and long-term survival is similar to other indications of liver transplantation [40, 41]. Patient should be managed conservatively with medical therapy while awaiting LT.

## 5. Conclusion

Pulmonary complications (HPS, PPH, and HH) are rare occurrence in patients with liver cirrhosis and portal hypertension. In patients with these conditions carry a significant morbidity and mortality and therefore, strong clinical suspicion is required to make earlier diagnosis. There are multiple medical therapies available for each condition in literature but most of the treatments are not effective. The only effective treatment that alters the clinical prognosis is liver transplantation and hence, patients with these conditions should be screened and assessed for the suitability of LT.

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Chapter 6

# Ascites: Causes, Diagnosis, and Treatment

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Additional information is available at the end of the chapter

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**Abstract**

Ascites is a pathological accumulation of fluid in the peritoneal cavity. Cirrhosis is the most common cause of ascites, representing for 85% of cases. More than one cause may be responsible for the development of ascites (multifactorial). Development of ascites is a poor prognostic event in the natural history of cirrhosis, with approximately 15 and 44% of patients with ascites succumbing in 1 and 5 years, respectively. Patients with cirrhosis need referral for liver transplantation after development of ascites. Proper history and physical examination are important in diagnosing the cause of ascites. Diagnostic paracentesis and abdominal sonogram should be performed during initial evaluation. Low salt diet and diuretic are the initial treatment option, and large volume paracentesis is an option for non-responder to diuretics. Transjugular intrahepatic portosystemic stent-shunt (TIPS) is highly valuable in properly selected patients.

**Keywords:** ascites, pathogenesis, diagnosis, diuretics, paracentesis, TIPS

## 1. Introduction

Ascites is defined as the pathological accumulation of excess fluid in the peritoneal cavity. Normally, the peritoneal cavity contains 25–50 mL of ascitic fluid, which allows for the movement of bowel loops past one other and helps hydrate serosal surfaces. With ascites, this fluid is not static within the peritoneal cavity, but is rather in a continuous exchange with the circulation through a large capillary bed under the visceral peritoneum, with about half the volume entering and leaving the peritoneal cavity every hour. Furthermore, the constituents of the fluid are in dynamic equilibrium with those of the plasma. However, the daily absorption of fluid from the peritoneal cavity back to the circulation is limited, and the maximum absorption of fluid out of the peritoneum is approximately 850 mL/d. Thus, the development of clinically significant ascites occurs when the rate of ascites formation exceeds the rate of

ascites reabsorption. For easily-controllable ascites, on the other hand, the volume of fluid that spills into the peritoneal cavity can be reduced below this absorption threshold. This is the case at the early stages of hepatic decompensating when ascites is responsive to a reduced intake of dietary sodium and to moderate doses of diuretics.

Cirrhosis is the most common cause of ascites, representing 85% of all cases of ascites [1]. In patients with cirrhosis, ascites due to portal hypertension (PHT) is primarily related to an inability to excrete adequate amounts of sodium into urine, leading to a positive sodium balance. Other causes of ascites include malignancy, heart failure, tuberculosis, alcoholic hepatitis, Budd-Chiari syndrome, and nephrogenic ascites [2]. More than one cause may be responsible for the development of ascites (multifactorial), such as the development of tuberculosis, heart failure, or peritoneal carcinomatosis in patients with cirrhosis and ascites [1]. Ascites is the most common complication of cirrhosis, as approximately 50% of patients with "compensated" cirrhosis develop ascites during 10 years of follow up [3]. The development of ascites is a poor prognostic event in the natural history of cirrhosis, with approximately 15% of patients succumbing in 1 year and 44% succumbing in 5 years [4]. Thus, these patients need to be referred for liver transplantation. Patients with cirrhosis and ascites are at high risk for other complications, including refractory ascites, spontaneous bacterial peritonitis (SBP), hyponatremia, or hepatorenal syndrome (HRS). The absence of these ascites-related complications qualifies ascites as uncomplicated [5]. Poor prognostic factors in patients with cirrhosis include hyponatremia, low arterial pressure, increased serum creatinine, and low urine sodium [6]. Among these factors, only serum creatinine is included in the Model for End-stage Liver Disease (MELD score) used for patient allocation for liver transplantation. Furthermore, serum creatinine has limitations in estimating glomerular filtration rate in cirrhosis [7], which usually underestimates the mortality risk in patients with ascites [8].

## 2. Pathogenesis of ascites in patients with liver cirrhosis

### 2.1. Pathogenesis and perpetuation of the ascites syndrome

Major factors involved in the complex pathogenesis of ascites are portal and sinusoidal hypertension, arterial vasodilatation, and neurohumoral activation, all leading to sodium and water retention [10, 11].

The pathogenesis of ascites is complex and not fully understood. The triad of portal hypertension, arterial vasodilatation, and neurohumoral activation, leading to sodium and water retention, explains, to large extent, the formation of ascites [9]. In fact, the direct cause of ascites formation in patients with cirrhosis is sodium retention, caused by decreased renal sodium excretion. The impairment in the renal ability to excrete sodium is considered the earliest manifestation of renal dysfunction in cirrhosis as shown by reduced natriuretic response to acute administration of sodium chloride [10]. Sodium retention in cirrhosis is mainly due to an increased tubular sodium reabsorption rather than decreased filtration of sodium. However, in the late stage of the disease, when hepatorenal syndrome develops,

sodium retention is caused by both increased reabsorption and decreased filtration. Sodium retention progresses with the advancement of liver disease; in the late stages of the disease, sodium retention becomes very high and the urinary sodium excretion may approach to zero. Sodium retention precedes the onset of ascites by few days, indicating that it is a cause and not a consequence of the accumulation of fluid within the abdominal cavity [10].

Portal hypertension (PHT) plays a major role in the development of ascites in patients with liver cirrhosis. The increased sinusoidal hydrostatic pressure and splanchnic capillary pressure are essential, and ascites usually develops in patients with a hepatic venous pressure gradient greater than 12 mmHg [11]. Patients with liver cirrhosis without portal hypertension do not develop ascites. In addition, lowering portal pressure in patients with cirrhosis and portal hypertension after surgical or radiological portosystemic shunts usually leads to better control of ascites. Sinusoidal or post sinusoidal portal hypertension is required for the development of ascites. On the other hand, presinusoidal hypertension alone, such as portal vein thrombosis (PVT), usually does not cause ascites unless associated with another contributing factor.

Additionally, portal hypertension results in increased level of vasodilator substances, e.g., nitric oxide (NO). This causes splanchnic and peripheral vasodilation and decreased effective blood volume leading to decreased renal blood flow and, subsequently, activation of the renin-angiotensin-aldosterone system (RAAS), sympathetic overactivity, and non-osmotic release of vasopressin [6, 12]. Renin is secreted from the renal juxtaglomerular apparatus secondary to changes in blood volume, changes in serum sodium, and increased activity of the sympathetic nervous system. In turn, renin will convert angiotensinogen to angiotensin I, which is then converted to angiotensin II by angiotensin-converting enzymes (ACE) in the lungs. Angiotensin II stimulates the release of aldosterone from the zona glomerulosa of the adrenal cortex [12]. Aldosterone stimulates sodium reabsorption in the distal tubule. Similarly, the renal sympathetic nervous activity stimulates sodium reabsorption in the proximal tubule, loop of Henle, and distal and collecting tubules. In patients with cirrhosis and portal hypertension, both the secondary hyperaldosteronism and the increased activity of the renal sympathetic nervous system play an important role in the pathogenesis of sodium retention. This excess sodium retention and the associated hypervolemia causing increased hydrostatic pressure will lead to excess transudation from both the hepatic sinusoids and the splanchnic capillaries, exceeding the re-absorptive capacity of the peritoneal surface and lymphatic system, which results in the development of ascites. Indeed, the formation of ascites depends on the balance between the hepatic sinusoidal and splanchnic filtration on the one hand and the lymph drainage on the other hand. Contrary to earlier theories, decreased plasma oncotic pressure has no role in the formation of ascites, and low plasma albumin level has little effect on the rate of ascites formation [13].

Furthermore, three theories of ascites formation have been proposed: underfilling, overflow, and peripheral arterial vasodilation (**Table 1**). The underfilling theory suggests that portal hypertension leads to increased filtration of fluid from the hepatic sinusoids and the splanchnic capillaries, leading to decreased effective circulating blood volume. This activates the plasma renin, angiotensin, aldosterone, and sympathetic nervous system, resulting in renal

| | |
|---|---|
| 1 | Under filling theory: increased filtration of fluid from the hepatic sinusoids and the splanchnic capillaries, leading to decreased effective circulating blood volume |
| 2 | Overflow theory: increased renal reabsorption of sodium unrelated to decreased blood volume |
| 3 | Peripheral arterial vasodilation: portal hypertension leads to vasodilation, which causes decreased effective arterial blood volume and hyperdynamic circulation |
| 4 | Renal resistance to atrial natriuretic peptide |

**Table 1.** Pathogenesis of ascites.

sodium and water retention. The overflow theory suggests that the primary abnormality is increased renal reabsorption of sodium unrelated to decreased blood volume. Several hypotheses that aim to explain this abnormality have been suggested including decreased hepatic synthesis of a natriuretic agent, decreased hepatic clearance of sodium retaining agent, or a primary hepatorenal reflex of unknown etiology. This overflow theory was supported by the observation that patients with cirrhosis have intravascular hypervolemia rather than hypovolemia, and sodium retention precedes ascites formation [14]. Nevertheless, both the underfill and overflow theories do not fully explain the formation of ascites and lack strong, supporting evidence. Finally, the arterial vasodilation hypothesis includes components of both the underfill and overflow theories. It suggests that portal hypertension leads to vasodilation, which causes decreased effective arterial blood volume and hyperdynamic circulation. This in turn activates neurohumoral systems leading to sodium retention and expansion of plasma volume, causing overflow of fluid into the peritoneal cavity. The theory also states that ascites formation is caused initially by underfilling of the intravascular compartment and is maintained by expansion of the intravascular compartment [12]. Moreover, the forward theory of ascites formation is a new modification of the vasodilation theory combining arterial underfilling with a forward increase in splanchnic capillary pressure and filtration with increased lymph formation [15].

Nitric oxide (NO) is the main vasodilator implicated in the systemic vasodilatation, and is primarily synthesized in the systemic vascular endothelium by NO synthase [16, 17]. Patients with portal hypertension have evidence of increased NO synthesis [18]. Calcitonin gene-related peptide (CGRP) and adrenomedullin are also potent vasodilatating factors, which have been found in increased levels especially in patients with ascites and hepatorenal syndrome (HRS) [18]. There is also evidence of increased resistance to vasoconstrictive substances, such as noradrenaline, angiotensin II, and vasopressin, which are most likely related to changes in receptor affinity, down-regulation of receptors, and to post-receptor defects related to increased NO expression [19]. Furthermore, alterations in vascular compliance is considered [20, 21], evidence show that it precedes neurohumoral activation and renal sodium and water retention [18].

Another mechanism that may contribute to ascites formation is renal resistance to atrial natriuretic peptide (ANP). ANP is a potent natriuretic peptide released from the cardiac atria in response to expansion of the intravascular volume. In compensated cirrhosis, ANP helps to maintain sodium balance by antagonizing the effect of antinatriuretic factors (aldosterone

and sympathetic overactivity). In later stages, renal resistance to ANP develops and leads to sodium retention [22].

The severity of renal sodium retention parallels the progression of cirrhosis due to the accentuation of the underlying vascular hemodynamic abnormalities and the associated activation of neurohumoral vasoactive mechanisms leading to avid renal reabsorption of sodium and water in the advanced stage of cirrhosis [15]. Furthermore, with progression of cirrhosis, renal perfusion and glomerular filtration rate progressively decline, leading to increased sodium reabsorption at the proximal convoluted tubule and decrease in its delivery to distal segments of the nephron [15]. Thus, in late stages of cirrhosis, renal sodium reabsorption mainly occurs proximal to the site of action of both the spironolactone and the loop diuretics rendering them ineffective. In addition, the increased resistance to vasoconstrictive substances, such as noradrenaline, angiotensin II, and vasopressin, accentuate the relative underfilling of the effective arterial blood volume, which aggravates the hypovolemic effects of diuretics, precluding the continuation of effective dosages of diuretics [23]. Accordingly, refractoriness to diuretic treatment is the end result of the accentuation of the hemodynamic abnormalities characterizing advanced cirrhosis. With further progression of liver disease and increased accentuation of these renal and vascular changes, these same mechanisms lead to hyponatremia and hepatorenal syndrome.

## 3. Evaluation of patients with ascites

The diagnosis of ascites is suspected based on the patient history and physical examination, and usually confirmed by abdominal ultrasound. The cause of ascites is identified based on the history, physical examination, laboratory tests, abdominal imaging, and ascitic fluid analysis. Patients with ascites usually present with abdominal distention, which may also be associated with abdominal discomfort, early satiety, weight gain, and shortness of breath. In addition, patients usually have symptoms and signs of the underlying cause of ascites. Since cirrhosis is the most common cause of ascites [1], history and physical examination should be directed for symptoms and signs of cirrhosis as well as risk factors for development of cirrhosis. Patients with cirrhosis may have other symptoms associated with hepatic decompensation, such as hepatic encephalopathy jaundice or gastrointestinal bleeding. Physical examination of patients with ascites due to liver cirrhosis usually reveals spider angioma, palmar erythema, jaundice, muscle wasting, gynecomastia, leukonychia, parotid enlargement, and abdominal wall collaterals. The liver and spleen may be palpable. Patients also need to be investigated for risk factors for cirrhosis including alcohol, viral hepatitis B and C, autoimmune liver disease, and other causes of cirrhosis. Those who lack an apparent cause for cirrhosis should also be questioned about lifetime body weight and diabetes as nonalcoholic steatohepatitis has been identified to be the cause of cirrhosis in many of these patients [24].

In addition to the clinical evaluation for cirrhosis, patients with ascites need to be evaluated for other causes including alcoholic hepatitis, heart failure, malignancy (peritoneal carcinomatosis, massive liver metastases, etc.), pancreatitis, nephrotic syndrome, tuberculous peritonitis,

acute liver failure, Budd-Chiari syndrome, and sinusoidal obstruction syndrome. Patients with malignant ascites may have symptoms related to the underlying malignancy, such as weight loss, whereas patients with ascites due to heart failure may have dyspnea, orthopnea, congested neck veins, and lower limb edema. Approximately 5% of ascites patients have 2 or more causes of ascites formation, i.e., "multifactorial" ascites. Most commonly, this presents as cirrhosis with another etiology as peritoneal tuberculosis. Laboratory test abnormalities seen in patients with ascites are related to the underlying cause of the ascites. Laboratory test abnormalities seen in patients with ascites are related to the underlying cause of the ascites. Patients with cirrhosis or heart failure usually have abnormal liver tests, increased serum bilirubin, hypoalbuminemia, elevated international normalized ratio (INR) in addition to thrombocytopenia, anemia, and leukopenia. Patients suspected of having ascites should have abdominal ultrasound to confirm the presence of ascites and to look for possible causes such as cirrhosis or malignancy. Ultrasound is probably the most cost-effective imaging modality. In patients with cirrhosis, ultrasound may reveal evidence of liver cirrhosis and portal hypertension including dilation of the portal vein to ≥13 mm, dilation of the splenic vein to ≥11 mm, reduction in portal venous blood flow velocity, splenomegaly (diameter >12 cm), and recanalization of the umbilical vein. Furthermore, ultrasound may also reveal evidence of hepatocellular carcinoma (HCC), which can be further evaluated with CT or magnetic MRI. Cardiac evaluation and echocardiography may also be needed to differentiate between cardiac ascites and cirrhotic ascites. Ascites due to cardiomyopathy can mimic that due to alcoholic cirrhosis. Pulmonary hypertension can also lead to heart failure and ascites. Jugular venous distension is present in the patients with cardiac ascites, but not in the ascites due to cirrhosis. Measuring the blood level of brain natriuretic peptide or pro-brain natriuretic peptide can help differentiating ascites due to heart failure (level usually about 6100 pg/ml) from ascites due to cirrhosis (166 pg/ml) [25].

## 4. Diagnostic paracentesis

Once the presence of ascites is confirmed, diagnostic paracentesis should be done to identify the cause of ascites and to rule out infection of the ascitic fluid. Abdominal paracentesis is indicated for all patients with new onset ascites [26]. Abdominal paracentesis is a safe procedure, and minor complications are rarely reported. The most common complication is abdominal wall hematomas, occurring in less than 1% of patients despite having abnormal prothrombin time in majority of cases [27]. This indicates that giving blood products such as platelets and fresh-frozen plasma before paracenteses is not needed [27, 28]. Routine tests of coagulation do not reflect bleeding risk in patients with cirrhosis; these patients usually have normal global coagulation because of a balanced deficiency of procoagulants and anticoagulants. Although more serious complications (hemoperitoneum or bowel entry by the paracenteses needle) may occur [28], they are rare (<1/1000 paracenteses) and should not deter the performance of this procedure. Bleeding complications occur mainly in patients with cirrhosis who have impaired renal function tests due to the associated platelet dysfunction in these patients [29]. Coagulopathy should preclude paracentesis only when there is clinically evident hyperfibrinolysis or clinically evident disseminated intravascular coagulation. A shortened euglobulin clot lysis time (<120 minutes) documents hyperfibrinolysis [30]. Epsilon aminocaproic acid

can be used to treat hyperfibrinolysis, and paracentesis can be performed after the lysis time has normalized on treatment [31].

## 5. Evaluation of ascitic fluid

The basic tests ordered on ascitic fluid samples include an analysis of the appearance, serum-to-ascites albumin gradient (SAAG), cell count and differential, culture, and total protein [26]. Fluid appearance can range from water-clear to frankly purulent, bloody, or chylous. The ascitic fluid cell count with the differential is the most important test performed on ascitic fluid to rule out infection since ascitic fluid infection is a treatable cause of deterioration as well as a preventable cause of death in patients with cirrhosis and ascites. Early diagnosis and proper treatment of ascitic fluid infection are crucial in patients with cirrhosis and ascites. Antibiotic treatment should be initiated in patients with a neutrophil count of ≥250/mm [32].

Culture of the ascitic fluid should be done in patients with new onset ascites, patients admitted to the hospital for ascites, in patients who develop fever or abdominal pain, and also in patients with cirrhosis who develop unexplained deterioration: increasing jaundice, azotemia, acidosis, or encephalopathy [32]. To increase the sensitivity of detecting bacterial growth in ascitic fluid, the ascitic fluid should be inoculated into blood culture bottles at the bedside; ascitic fluid culture is positive in only 50% of patients with spontaneous bacterial peritonitis (SBP) by older methods, compared to approximately 80%, if the fluid is inoculated into blood culture bottles at the bedside and prior to administration of antibiotics [33, 34]. A single dose of an effective antibiotic usually leads to a negative bacterial culture [35].

Initially, ascitic fluid was classified as an exudate or transudate based on total protein concentration. Recently, this exudate/transudate classification has been replaced by the SAAG, which is a more useful measure for determining the presence or absence of portal hypertension [1, 36]. However, the ascitic fluid total protein concentration remains of some value as patients with an ascitic fluid protein of <1 g/dL have a high risk of SBP requiring prophylactic antibiotics [37]. The SAAG is easily calculated by subtracting the ascitic fluid albumin value from the serum albumin value, which should be obtained the same day. The SAAG accurately identifies the presence of portal hypertension; SAAG ≥1.1 g/dL (≥11 g/L) predicts that the patient has portal hypertension with 97% accuracy, while SAAG <1.1 g/dL (<11 g/L) indicates that the patient does not have portal hypertension [1].

While SAAG in patients with ascites due to heart failure can be affected with diuretics, the SAAG in the setting of cirrhosis remains stable unless portal pressure decreases significantly [38]. If the results of these tests are abnormal, further testing can be performed on another ascitic fluid sample. These additional ascitic fluid tests are requested based on the clinical scenario. The following is a list of tests that can be conducted to test for ascites.

- Glucose concentration: White blood cells, bacteria, and malignant cells consume glucose; thus, the concentration of glucose may be low in peritoneal carcinomatosis and bowel perforation [35, 39].

- Lactate dehydrogenase (LDH) concentration: The ascitic fluid/serum (AF/S) ratio of LDH is about 0.4 in cirrhotic ascites without infection. In SBP, the ascitic fluid LDH level rises such that the ascitic fluid/serum (AF/S) ratio of LDH approaches 1.0. In the case of bowel perforation, or peritoneal carcinomatosis, the ascitic fluid/serum (AF/S) ratio of LDH is greater than 1.0 [40].
- Gram stain: The sensitivity of ascitic fluid gram stain is only 10%. The main benefit of gram stain of ascitic fluid is to differentiate between SBP and bowel perforation where there is polymicrobial growth in bowel perforation and monomicrobial growth in SBP [41].
- Amylase concentration: The ascitic fluid amylase concentration is increased in pancreatitis or bowel perforation reaching approximately 2000 unit/L [42].
- Tests for tuberculous peritonitis: A variety of tests have been used for the detection of tuberculous peritonitis including direct smear, culture, cell count with predominance of mononuclear cells, and adenosine deaminase. Only patients at high risk for tuberculous peritonitis should have testing for mycobacteria on the first ascitic fluid specimen. The sensitivity of smear of ascitic fluid for mycobacteria is almost zero [43], while the sensitivity of fluid culture for mycobacteria reaches 50% [44]. Polymerase chain reaction testing for mycobacteria, laparoscopy with biopsy, and mycobacterial culture of tubercles are the most rapid and accurate methods of diagnosing tuberculous peritonitis [45].
- Cytology: It should be requested only if malignant ascites is suspected. The sensitivity of ascitic fluid cytology in peritoneal carcinomatosis is approximately 100% [46]. However, because not all cases of malignant ascites are associated with peritoneal carcinomatosis, the overall sensitivity of cytology smears for the detection of malignant ascites is 58–75% [47]. Hepatocellular carcinoma (HCC) rarely metastasizes to the peritoneum.
- Triglyceride concentration: Chylous ascites has a triglyceride content greater than 200 mg/dL (2.26 mmol/L) and usually greater than 1000 mg/dL (11.3 mmol/L) [48].
- Bilirubin concentration: Ascitic fluid bilirubin value greater than the serum suggests bowel perforation or biliary leak [49].

## 6. Treatment of ascites

Proper management depends on the cause of ascites. Patients with high SAAG (portal hypertensive) ascites usually respond to dietary salt restriction and diuretics. Conversely, patients with low SAAG ascites (with the exception of nephrotic ascites) do not respond to dietary salt restriction and diuretics; treatment of ascites in these patients depends on successful treatment of the underlying disorder. Improvement of cirrhosis alone can lead to control of ascites and better response to diuretics. This is particularly true for patients with alcoholic liver disease [50], Hepatitis B virus (HBV)-related liver disease [51], and autoimmune hepatitis, where specific treatment of cause of cirrhosis by ceasing alcohol consumption, HBV antiviral therapy, or immunosuppression can lead to regression of cirrhosis and better control of ascites.

The approach for the treatment of ascites depends on the grade of ascites. According to the International Ascites Club, ascites is classified into three grades according to the severity of ascites [5].

Grade 1—Mild ascites detectable only by ultrasound examination.

Grade 2—Moderate ascites with moderate abdominal distension.

Grade 3—Marked ascites with marked abdominal distension.

Currently, there are no recommendations for the treatment of grade 1 ascites. Grade 2 ascites can be treated with dietary sodium restriction and diuretics. Grade 3 ascites can be treated with initial large volume paracentesis followed by dietary sodium restriction and diuretics [52].

## 7. First-line therapy for ascites

### 7.1. Dietary sodium restriction

The first-line treatment of patients with cirrhosis and ascites is dietary sodium restriction (2000 mg per day [88 mmol per day]) [53]. This is generally equivalent to a no added salt diet, and avoiding pre-prepared meals. More strict sodium restriction may improve mobilization of ascites, although it is not recommended because it is less palatable and may worsen the already existing malnutrition in patients with cirrhosis. Total non-urinary sodium excretion is less than 10 mmol per day in afebrile patients with cirrhosis without diarrhea [54]. Based on that, ascites can be controlled if urinary excretion of sodium exceeds 78 mmol per day (88 mmol intake per day − 10 mmol nonurinary excretion per day) in patients on restricted sodium diet. However, only 10–15% of patients have urinary excretion of sodium greater than 78 mmol per day and only those patients can be considered for dietary sodium restriction alone. Measurement of urinary sodium excretion is a helpful parameter to assess compliance with dietary sodium restriction. Patients with urinary excretion of sodium greater than 78 mmol per day without improvement of ascites are not compliant with salt restriction. Urinary sodium excretion can be measured by random urinary sodium concentrations, 24-hour urinary sodium or urine sodium/potassium ratio.

### 7.2. Diuretics

Renal sodium retention in the setting of liver cirrhosis and ascites is due to increased proximal and distal tubular reabsorption of sodium [55, 56]. The mechanism of increased proximal tubular reabsorption of sodium is not completely understood, while the increased sodium reabsorption in the distal tubule is due to hyperaldosteronism [55]. In patients with liver cirrhosis, secondary hyperaldosteronism is a major factor promoting renal sodium retention in the distal tubules and collecting ducts of the nephron. Clinical trials have shown that spironolactone is the drug of choice for the initial treatment of ascites. Spironolactone achieves a better natriuresis than “loop” diuretics in cirrhotic patients with ascites [56]. Although spironolactone

is effective for mobilization of ascites, most patients will eventually require both diuretics. Furthermore, starting with both drugs is more effective in achieving rapid mobilization of ascites and maintaining normokalemia [57, 58].

The initial doses of both diuretics are 100 mg/d for spironolactone and 40 mg/d for furosemide. If inadequate, the dose can be increased every 3–5 days to a maximum dose of 400 mg aldactone and 160 mg of furosemide [53]. The target of diuretic therapy is to achieve 0.5 kg/day weight loss in patients without peripheral edema and up to 1 kg/day in patients with peripheral edema while monitoring renal function and sodium [59]. Furosemide can be temporarily withheld in patients presenting with hypokalemia; this is very common in the setting of alcoholic hepatitis. Patients with parenchymal renal disease or post liver transplantation may tolerate less spironolactone than usual because of hyperkalemia. Single morning dosing maximizes compliance. Dosing more than once daily reduces compliance and can cause nocturia. The use of diuretics may be associated with several complications such as renal failure, electrolyte disorders, muscle cramps, and hepatic encephalopathy [30, 31, 55–57, 59–63].

Gynecomastia is the main side effect of spironolactone, but metabolic acidosis with or without hyperkalemia may also occur in patients with renal impairment. Other side effects of furosemide include potassium depletion, metabolic hypochloremic alkalosis, and hyponatremia, as well as hypovolemia, leading to renal dysfunction. The use of intravenous furosemide is not recommended, as it may cause an acute reduction in renal perfusion and subsequent azotemia in patients with cirrhosis and ascites. Amiloride (10–40 mg per day) is another aldosterone antagonist and can replace spironolactone in patients with tender gynecomastia. However, amiloride is more expensive and has been shown to be less effective than spironolactone [61]. Triamterene, metolazone, and hydrochlorothiazide have also been used to treat ascites [64].

Hydrochlorothiazide can also cause rapid development of hyponatremia when added to the combination of spironolactone and furosemide; it should be used with extreme caution or avoided entirely.

While patients are on diuretics, monitoring of body weight, blood pressure, orthostatic symptoms, and serum electrolytes, urea, and creatinine levels needs to be checked regularly. If weight loss is inadequate, assessment of urinary sodium excretion needs to be done by urine sodium/potassium ratio or by 24-hour urine sodium. Patients who are excreting urine sodium/potassium greater than 1- or 24-hour urine sodium greater than 78 mmol per day and not losing weight are not compliant with dietary sodium restriction. These patients should not be labeled as diuretic-resistant that require second-line therapy. On the other hand, in patients who are not losing weight and their urinary sodium excretion is less than 1- or 24-hour urine sodium less than 78 mmol per day, the dose of diuretic needs to be increased gradually [26]. Following mobilization of ascites, diuretics should be reduced to maintain patients with minimal or no ascites to avoid diuretic-induced complications.

In patients with ascites and lower limb edema, there is no limit for daily weight loss due to the use of diuretics because there is no limit for mobilization of fluid from the interstitial fluid to the vascular compartment [65]. However, in patients with ascites and no lower limb edema, daily weight loss of 0.5 kg is a reasonable daily maximum as this is likely the maximum daily

mobilized fluid from ascites to the vascular compartment. Diuretics should be stopped if the patient has hepatic encephalopathy, rising serum creatinine (>2.0 mg/dl) while on diuretics or if there is hyponatremia (<120 mmol/L) not corrected with fluid restriction [26].

Dietary sodium restriction and a dual diuretic regimen with spironolactone and furosemide have been shown to be effective in more than 90% of patients in achieving a reduction in the volume of ascites to acceptable levels [58]. Less than 10% of patients with cirrhosis and ascites are refractory to standard medical therapy [30, 56–58, 66, 67].

Patients with liver cirrhosis are in a state of systemic and splanchnic vasodilatation caused by nitric oxide and other vasodilators. Blood pressure is maintained in these patients due to the compensatory increased levels of vasopressin, angiotensin, and aldosterone and sympathetic overactivity [68]. The use of drugs, which decrease the level or antagonize the effect of these hormones, is expected to lower blood pressure and affect survival of those patients. These include angiotensin converting enzyme inhibitors, angiotensin receptor blockers, and beta blockers [69]; these drugs should be avoided in patients with ascites and in the rare situation where the benefit of using these drugs overweighs their risks, and blood pressure and renal function must be monitored carefully to avoid rapid development of renal failure.

Other drugs that should be avoided in patients with ascites are Prostaglandin inhibitors such as nonsteroidal anti-inflammatory drugs. These drugs antagonize the vasodilator effect of prostaglandins on renal artery causing reduction of urinary sodium excretion and can also cause azotemia [70]. Only unusual patients whose risk of an ischemic cardiac or neurologic event exceeds the risk of worsening azotemia or gut bleeding should take low dose aspirin.

### 7.3. Single large volume paracentesis (LVP)

Large volume paracentesis is associated with circulatory dysfunction called post paracentesis circulatory dysfunction (PPCD). It leads to complication in patients with liver cirrhosis including rapid accumulation of ascites [71–74], development of HRS and/or water retention leading to dilutional hyponatremia [72], further increase of portal pressure [75], and shortened survival [73]. The most effective method to preventing circulatory dysfunction after LVP is the administration of albumin [73]. Large volumes of fluid have been safely removed with the concomitant administration of intravenous albumin (6–8 g/L of fluid removed) [76]. However, single 5-L paracentesis can be performed safely without albumin infusion [77]. LVP with albumin is the best treatment option in patients with grade 3 ascites; it is more effective and safer than diuretics as it is associated with less hyponatremia, renal impairment, and hepatic encephalopathy. There were no differences between the two approaches with respect to hospital readmission or survival [71–73, 78–81]. LVP is a safe procedure, and the risk of local complications, such as hemorrhage or bowel perforation, is extremely low [29].

Additionally, although paracentesis removes the fluid more rapidly than does careful diuresis, paracentesis does nothing to correct the underlying problem that led to the initial ascites formation, i.e., sodium retention, and it should not be viewed as first-line therapy for all patients with ascites. Dietary sodium restriction and diuretics should follow paracentesis to prevent or decrease fluid re-accumulation.

## 8. Refractory ascites

Refractory ascites is defined as ascites that is unresponsive to a sodium-restricted diet and high doses of diuretics or recurs rapidly after therapeutic paracentesis [82]. Refractory ascites is classified as diuretic-resistant ascites when there is poor control of ascites as well as low urinary sodium excretion (<78 mmol/d), despite maximal diuretics or diuretic intractable ascites, where the use of high-dose diuretics is not applicable due to development of clinically significant complications of diuretics [5]. Once the patient is considered diuretic-resistant, diuretics should be discontinued and these patients will need second-line therapy. The European guideline recommends discontinuing diuretics if the urine sodium is <30 mmol/day during diuretic therapy. Oral midodrine 7.5 mg three times daily has been shown to increase urine volume, urine sodium, mean arterial pressure, and survival in patients with refractory ascites. Midodrine can be added to diuretics to increase blood pressure and theoretically convert diuretic-resistant patients back to diuretic-sensitive [83]. Once ascites becomes refractory to medical treatment, the median survival of patients is approximately 6 months [82, 84–86].

Hence, patients with refractory ascites should be considered for liver transplantation. The MELD score system which predicts survival in patients with cirrhosis [87, 88] does not include low arterial pressure, low serum sodium, low urine sodium, or Child-Turcotte-Pugh (CTP) score, all of which are important prognostic factors [84–88]. Consequently, patients with refractory ascites may have a poor prognosis despite a relatively low MELD score (e.g., <18). For these reasons, inclusion of additional parameters in the MELD score, such as serum sodium, is suggested [88–90].

## 9. Second-line therapy for ascites

Patients with refractory ascites who do not respond to first-line therapy of dietary sodium restriction and diuretics may benefit from second-line therapy. Second-line therapy for ascites includes serial therapeutic paracenteses, transjugular intrahepatic portosystemic stent-shunt (TIPS), peritoneovenous shunt (PVS), and liver transplantation.

### 9.1. Serial therapeutic paracenteses

Serial paracenteses is a safe option for patients with refractory ascites. Large volume paracenteses up to total paracenteses can be done on regular basis or in demand. Diuretics can be stopped in these patients, especially if urine sodium is still <30 mmol/day, but dietary sodium restriction should be maintained to decrease the rate of fluid accumulation. The frequency of paracenteses depends on the patient's compliance with the low-sodium diet. Patients who need more frequent taping than 10 L every 2 weeks are not compliant with diet. Paracentesis of large volume of ascitic fluid is associated with changes in electrolytes, plasma renin, aldosterone, and angiotensin levels and may also develop acute rise of serum creatinine [72–74]. An albumin infusion of 6–8 g/L of fluid removed given during paracenteses, or shortly after, abolishes these hormonal changes and appears to improve survival [73]. Up to 5 L of ascites can be taped safely without the need for albumin infusion [77]. An alternative approach with

similar efficacy to albumin infusion is intravenous terlipressin (1 mg at onset of paracentesis, 1 mg at 8 hours, and 1 mg at 16 hours) as well as midodrine orally (for 72 hours after paracentesis) [83, 91].

### 9.2. Transjugular intrahepatic portosystemic stent-shunt (TIPS)

TIPS is a side-to-side portosystemic shunt created between the portal vein and the hepatic vein via intrahepatic self expandable stent [92–96]. TIPS can achieve portal decompression, and therefore prevention of complications of portal hypertension such as variceal bleeding, ascites, and hydrothorax. Additionally, TIPS increases glomerular filtration and urine output, promotes natriuresis, and reduces the plasma renin activity, aldosterone, and noradrenaline levels causing improvement of renal dysfunction related to the circulatory and hormonal changes in cirrhotic patients [97–99]. The main indication for TIPS is refractory ascites, uncontrolled acute variceal bleeding, and secondary prevention of gastric variceal bleed. It may have a role in hydrothorax, hepatorenal, and hepatopulmonary syndrome [100].

Early studies comparing TIPS with large volume paracentesis were disappointing. Despite better control of ascites in patients undergoing TIPS, there was no survival advantage in TIPS in addition to increased morbidity due to hepatic encephalopathy and deterioration of liver function [94]. This can be explained by poor patient selection in early experience with TIPS. However, in the meantime, better selection of patients for TIPS together with the use of polytetrafluorethylene (PFTE)-covered stents resulted in high response rate comparable with surgical shunts. The good results of TIPS obviate the need for surgical shunt [101, 102]. Recent studies had shown that TIPS is not only more effective in control of ascites than repeated large volume paracentesis but also improves survival [92, 93, 95, 96].

The main complication of TIPS is the development of hepatic encephalopathy which is more reported with TIPS than with repeated large volume paracentesis [103–107]. Other complications include shunt thrombosis and stenosis. Uncovered stents are complicated by stenosis in up to approximately 80% of cases [11, 108]. TIPS usually converts diuretic-resistant patients into diuretic-sensitive patients, therefore diuretics and dietary salt restriction must be started in these patients to maintain control of ascites. Absolute and relative contraindication to TIPS insertion includes congestive heart failure, severe pulmonary hypertension, severe hepatic decompensation, recurrent portosystemic encephalopathy, polycystic liver disease, hepatic abscess, and hepatocellular carcinoma [100].

## 10. Third-line therapy for ascites

### 10.1. Peritoneovenous shunts

The peritoneovenous shunt (PVS) has been widely used as a suitable alternative to repeated large volume paracentesis in patients with refractory ascites [109]. The negative pressure in the chest allows fluid to move from the high-pressure intraperitonium to the chest through the one-way valve tube through subcutaneous tissue of the chest wall to the internal jugular vein to the superior vena cava. Among the various complications associated with PVS, the

most common one is the obstruction of the prosthesis, which occurs in 40–60% of patients during first year of follow-up [110]. This procedure has a very limited use due to high complication rate, low long-term patency rate without survival advantage [58, 111]. However, it can be used in patients with refractory ascites who are not candidate for TIPS or liver transplant or for serial paracenteses because of multiple abdominal scars or distance from a physician willing and capable of performing paracenteses (**Table 2**).

| | |
|---|---|
| First line | - Dietary sodium restriction |
| | - Diuretic |
| | - Single large volume paracentesis |
| Second line | - Serial therapeutic paracenteses |
| | - Transjugular intrahepatic portosystemic stent-shunt |
| Third line | - Peritoneovenous shunts |

**Table 2.** Treatment of ascites.

## 11. Conclusion

Liver cirrhosis is the main cause of ascites; ascites in the setting of liver cirrhosis is caused by portal hypertension that leads to vasodilation, with decreased effective arterial blood volume and hyperdynamic circulation. SAAG and ascitic fluid cell count are an important diagnostic tools.

The first-line therapy is low salt diet and diuretics, which is effective in nearly 90% of patients, LVP with albumin is the best treatment option in patients with intractable ascites, and TIPS can be used in selected patients with good results. Surgical shunt for ascites is almost obsolete.

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Chapter 7

# Nutritional Status in Liver Cirrhosis

Kazuyuki Suzuki, Ryujin Endo and Akinobu Kato

Additional information is available at the end of the chapter

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**Abstract**

The metabolism of many nutritional elements (carbohydrate, protein, fat, vitamins, and minerals) is gradually disturbed with progressive chronic liver diseases. In particular, protein-energy malnutrition (PEM) is known as the most characteristic manifestation of liver cirrhosis (LC) and is closely related to its prognosis. Recently, while sarcopenia (loss of muscle mass and strength or physical performance) has been discussed as an independent factor associated with prognosis in patients with LC, obesity and insulin resistance in patients with LC also contribute to carcinogenesis in LC. Deficiencies of zinc and carnitine are involved in the malnutrition in LC and are associated with hyperammonemia, which is related to the pathogenesis of hepatic encephalopathy. Because the nutritional and metabolic disturbances in LC are fundamentally influenced by many factors, such as the severity of liver damage, the existence of portal-systemic shunting, and inflammation, proper nutritional assessment is necessary for the nutritional management of patients with LC.

**Keywords:** liver cirrhosis, malnutrition, protein-energy malnutrition, sarcopenia, glucose intolerance

## 1. Introduction

The liver plays a central role in the metabolism of many nutritional elements (carbohydrate, protein, fat, vitamins, and minerals). The metabolism of these nutritional elements is gradually disturbed with progressive chronic liver disease. Protein-energy malnutrition (PEM) is the most characteristic manifestation and is closely related to the prognosis and the quality of life in liver cirrhosis (LC) [1–7]. PEM can lead to muscle atrophy and reduced strength [8–12],

which is defined as sarcopenia and has recently been considered an independent prognostic factor in LC with PEM [13–16], while overweight or obesity has been seen as one of the important factors related to carcinogenesis in LC [17]. The relationships among PEM, sarcopenia, and prognosis in LC are shown in **Figure 1**. Furthermore, glucose intolerance or diabetes mellitus (DM) is also an independent factor related to carcinogenesis in LC [18–23]. Serum zinc (Zn) and carnitine (CA) status are involved in the malnutrition in LC and are associated with hyperammonemia, which is related to the pathogenesis of hepatic encephalopathy (HE) [24–31].

Malnutrition in LC is affected by many factors, such as the severity of liver damage, the existence of portal-systemic shunting, and inflammation [10, 32]. Therefore, for the proper nutritional management of patients with LC, precise nutritional assessment is needed.

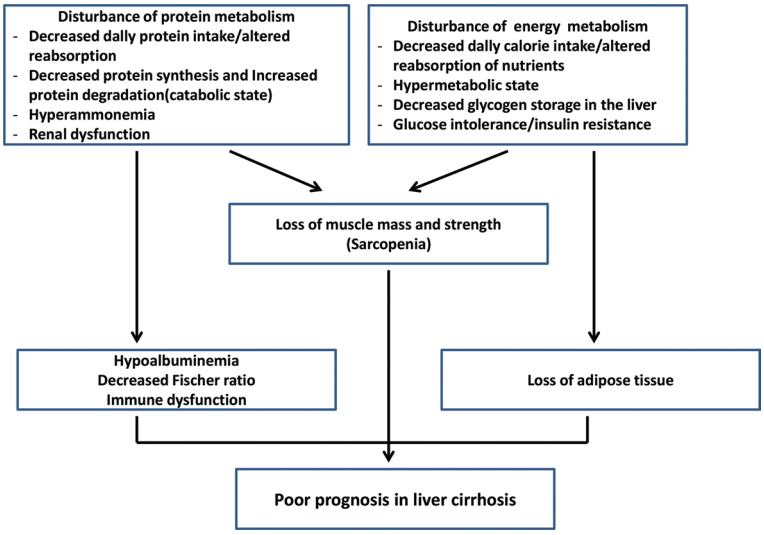


**Figure 1.** Relationships among protein-energy malnutrition, sarcopenia, and prognosis in liver cirrhosis patients.

This chapter focuses on the association between nutritional assessment and malnutrition in patients with LC.

## 2. Nutritional assessments

Recommended nutritional assessments in patients with LC are shown in **Table 1**. Static and dynamic status of nutrition should be necessary. Dietary assessment by a skilled dietitian is the first step in assessing nutritional status. Simple and easy applied methods, such as the subjective global assessment (SGA), mini nutritional assessment (MNA), and anthropometric parameters, are recommended in the assessment of nutritional status [32]. Biomarkers representing serum albumin (Alb) are important to assess nutritional status. However, because many biomarkers

| | |
|---|---|
| **1. Static status of nutrition** | |
| **a.** | Daily food intake |
| **b.** | Body composition analysis<br>Height, body weight, body mass index, anthropometric parameters,<br>bioelectrical impedance analysis (BIA) |
| **c.** | Biomarkers<br>Red blood cell count, hemoglobin, routine liver function tests, cholesterol, cholinesterase, albumin, rapid turnover proteins, adipocytokines (adiponectin, leptin, resistin, etc.), tumor necrosis factor-$\alpha$, ghrelin, vitamins, minerals, creatinine height index in urine |
| **d.** | Immune reaction<br>Total lymphocyte count, delated cutaneous hypersensitivity, purified protein derivate of tuberculin |
| **e.** | Imaging<br>Computer tomography (abdomen) |
| **2. Dynamic status of nutrition** | |
| **a.** | Energy metabolism using indirect calorimetry |
| **b.** | Nitrogen balance |
| **c.** | Biomarkers: plasma free amino acids pattern (Fischer ratio and BTR*) |
| **d.** | Urinary 3-methylhistidine excretion |

*Fischer ratio, branched chain amino acids (BCAA)/phenylalanine + tyrosine; BTR, BCAA/tyrosine ratio.

**Table 1.** Recommended nutritional assessment in patients with liver cirrhosis.

are often affected by complications such as infection and renal dysfunction, the data must be carefully interpreted. Energy metabolism assessment (e.g., resting energy expenditure (REE), nonprotein respiratory quotient (npQR), and substrate oxidation rates for glucose, protein, and fat) using indirect calorimetry is the most useful method to assess whether patients with LC have PEM [32–35]. However, this method cannot be used routinely and easily to examine outpatients, because the indirect calorimeter has a high cost, and it takes time to perform the test.

### 2.1. Changes of body composition

Analysis of body composition includes height, body weight, body mass index (BMI), and anthropometric parameters. Anthropometric parameters include percent ideal body weight (IBM), triceps skin fold thickness (TSF), arm circumference (AC), and arm muscle circumference (AMC). Among these parameters, TSF and AMC are significantly correlated with muscle volume or the volume of total body fat mass [34, 35]. However, these parameters cannot be accurately estimated in patients with LC who have edema and/or ascites. Recently, new methods of body mass composition analysis using computer tomography and bioelectrical impedance analysis have been developed in daily clinical practice, but this method also cannot provide accurate results in patients with LC who have edema and/or ascites [12–14].

In various chronic liver diseases including LC, several previous reports have shown skeletal muscle loss using anthropometric parameters [1–4, 11]. This status has recently been defined

as sarcopenia, which shows loss of muscle mass and muscle strength or physical performance [8–12]. Although multiple factors, including differences in the etiology of LC, duration of disease, and the severity of liver damage, are related to the prevalence of sarcopenia in LC, sarcopenia is seen in approximately 30–70% of patients with LC [11–14, 35]. Additionally, a recent study showed that sarcopenia is a risk factor for recurrence in LC patients with hepatocellular carcinoma who undergo curative treatment [14].

Muscle mass is the result of a dynamic balance between protein synthesis and degradation [36–39]. This balance is regulated by two major branches of AKT (also known as protein kinase B) signaling pathways: the AKT/mammalian target of rapamycin (mTOR) pathway that controls protein synthesis and the AKT/forkhead box O (FOXO) pathway that controls protein degradation. Recent reports have shown that myostatin, a member of the transforming growth factor-β superfamily, has emerged as a key regulator of skeletal muscle mass [39]. Myostatin is also a key mediator between energy metabolism and endurance capacity of skeletal muscle [37–39].

On the other hand, the prevalence of LC patients with obesity has increased in the last decade [17]. The definition of obesity is different between Japan and European countries (body mass index (BMI) ≥ 25 kg/m$^2$ in Japan and ≥30 kg/m$^2$ in European countries). Obesity in patients with LC is associated with insulin resistance, which has been discussed as an important factor in carcinogenesis in LC [17–22].

### 2.2. Changes of biomarkers

Serum Alb is a main secretion protein synthesized by the liver and has multiple functions, such as the maintenance of colloid osmotic pressure, ligand binding and transport, and enzymatic and antioxidative activities [40, 41]. The synthesis and degradation rates of Alb in patients with LC are decreased compared with those in healthy individuals whose liver function is normal. In particular, the half-life of serum Alb is extended in patients with LC [42]. The serum Alb concentration is affected by the volume of daily food intake, digestion and absorption from the intestine, the degree of severity of liver damage, the imbalances of various hormone dynamics, and nutritional and catabolic status, such as that conferred by infections and burns [43]. However, serum Alb concentration is still frequently used as a biomarker of malnutrition and as an item of both the Child-Pugh classification score and the modified end-stage liver disease (MELD) score [44, 45]. Serum Alb is microheterogeneous with oxidized and reduced forms. Serum Alb concentration decreases, while the ratio of oxidized Alb increases, with LC progression [46, 47]. A recent report has shown that this ratio improved in patients with LC after supplemental treatment with a branched-chain amino acid (BCAA; valine, leucine, and isoleucine)-enriched formula [48]. These findings suggest that the oxidative status of serum Alb could provide a better assessment of malnutrition, though the measurement of serum levels of oxidized and reduced forms of Alb is time-consuming and inconvenient in the clinical setting.

Rapid turnover proteins such as transthyretin (prealbumin), retinol-binding protein, and transferrin are useful biomarkers of short-term nutritional status in patients with LC. The half-life time is 2 days for transthyretin, 0.4–0.7 days for retinol-binding protein, and 7–10 days for transferrin [49, 50]. These proteins are also influenced by baseline conditions such as surgery, infection, and

anemia [50]. Recent reports have suggested that serum retinol-binding protein 4 (RBP-4) is a biomarker for assessing malnutrition in patients with LC. Serum RBP-4 levels are decreased in patients with LC and directly related to the severity of liver damage according to the Child-Pugh classification, while these levels are not correlated with insulin resistance [51, 52].

The profiles of plasma amino acids show characteristic changes in patients with LC. In particular, the plasma concentration of BCAAs is decreased, while that of aromatic amino acids (AAA; phenylalanine (Phe) and tyrosine (Tyr)) is increased, resulting in a decreased BCAA/ AAA molar ratio (namely, the Fischer ratio) or the BCAA/Tyr ratio (BTR) [53–55]. BCAA is mainly metabolized and used to detoxify ammonia and for energy production in the skeletal muscle. AAA is metabolized in the liver and is a representative precursor of a neurotransmitter (dopamine) and a pseudo-neurotransmitter (octopamine), which are closely associated with the pathogenesis of HE [53]. The plasma Fischer ratio and serum BTR are significantly correlated with the serum Alb concentration and the severity of liver damage according to the Child-Pugh classification (**Figure 2**), but not with the degree of HE [32, 55]. Furthermore, serum BTR can help predict a decrease in serum Alb concentration associated with chronic liver diseases [56].

Adipocytokines are also biomarkers of nutritional status in patients with LC. Leptin, adiponectin, and resistin are representative peptide hormones that are produced by adipose tissue, and they are closely associated with insulin resistance and arteriosclerosis [32]. Serum leptin levels are higher in females than males among healthy individuals and patients with LC. These levels are correlated with AMC and TSF, but they are not correlated with the severity of liver

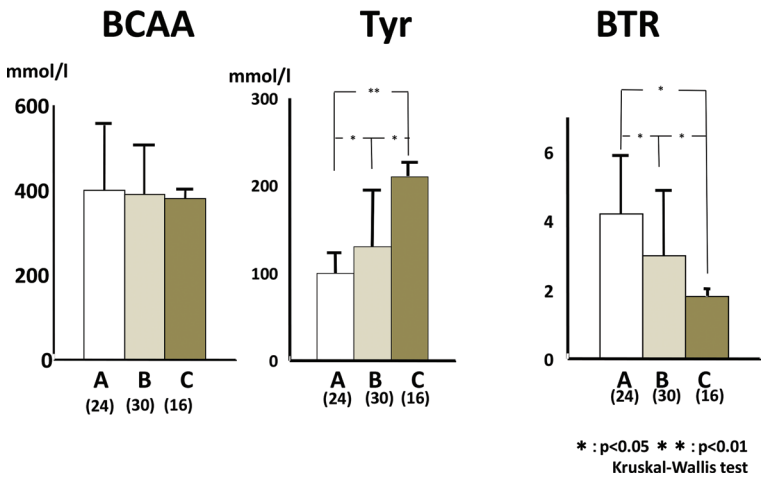


**Figure 2.** Plasma branched-chain amino acids, tyrosine, and the branched-chain amino acid to tyrosine ratio in patients with liver cirrhosis. Seventy cirrhotic patients with or without hepatocellular carcinoma who were admitted to Iwate Medical University Hospital were investigated. Serum amino acid concentrations were measured by an enzymatic method. The severity of liver damage was classified into grades A, B, and C based on the Child-Pugh classification. BCAA, branched-chain amino acid (valine + leucine + isoleucine); BTR, BCAA/tyrosine ratio. Each value is shown as the mean ± standard deviation. *P < 0.05, **P < 0.01 (Kruskal-Wallis test). (), number of patients with LC.

damage [57–59]. Plasma adiponectin assumes three forms: low molecular weight, medium molecular weight, and high molecular weight [60–62]. In patients with LC, the high molecular weight form of plasma adiponectin is significantly increased compared with healthy individuals and is correlated with the severity of liver damage [32, 62]. Plasma resistin levels associated with insulin resistance are also correlated with the severity of liver damage in patients with LC [63, 64].

Ghrelin, an orexigenic hormone and stimulator of growth hormone, is mainly found in the gastric wall [65, 66]. Ghrelin plays a role in the hypothalamic centers to regulate feeding and caloric intake [65–67]. Furthermore, ghrelin controls feeding behavior and the long-term regulation of body weight in association with leptin in the hypothalamic centers [66, 67]. The plasma ghrelin level has been considered a marker of pathological conditions such as obesity, insulin resistance, type 2 DM, and hypertension. However, the plasma ghrelin level in patients with LC was controversial in previous reports [68–70]. Our study has shown that the plasma ghrelin level (desacyl form) is higher in LC patients than in healthy controls, while it is not correlated with the severity of liver damage. Rather, the plasma ghrelin level is significantly correlated with BMI, AMC, TSF, and non-protein respiratory quotient (npRQ) [70].

Vitamins (fat-soluble: A, D, E, and K, and water-soluble: thiamine, riboflavin, niacin, $B_6$, $B_{12}$, C, and folate), carnitine (CA), minerals, trace elements (copper, zinc, iron, manganese, and selenium), and hormones (insulin-like growth factor 1, insulin-like growth factor-binding protein 3, reverse triiodothyronine, etc.) need to be examined when assessing the nutritional status of LC patients. In particular, evaluations of serum zinc and CA (total CA, free CA, and acyl-CA) are necessary in LC patients with sarcopenia and hyperammonemia [23–32].

### 2.3. Disturbances of energy metabolism

PEM is a characteristic state of malnutrition in advanced LC and is closely associated with the survival rate, the carcinogenic risk, and the outcome of liver transplantation in patients with LC. The serum Alb concentration is generally a marker of protein malnutrition. The npRQ using indirect calorimetry is a marker of energy malnutrition [71]. Therefore, indirect calorimetry would be the best method to assess PEM. The results of REE, npRQ, and the oxidation rates of three nutrients (carbohydrate, protein, and fat) are obtained by indirect calorimetry. Many previous reports indicated that the npRQ decreases, the oxidation rate of fat increases, and the oxidation rate of carbohydrate decreases according to the Child-Pugh classification [5, 72, 73]. It has been considered that a decreased npRQ (<0.85) after an overnight fast predicts a catabolic state and is related to a lower survival rate in LC patients [5]. Decreased carbohydrate oxidation is explained by both the lower production rate of glucose from glycogen in the liver and decreases in peripheral glucose use due to insulin resistance [74]. In fact, patients with LC cannot store sufficient glycogen due to liver atrophy, and their energy generation pattern after an overnight fast is equivalent to that observed in healthy individuals after 2–3 days of starvation [74, 75]. Increased fat oxidation is caused by an increased rate of lipolysis in fat tissue [76]. Our earlier results are generally similar to previous reports (**Figures 3** and **4**). However, because measurement by indirect calorimetry is not easy, it cannot be routinely performed in outpatients with LC. The serum free fatty acid (FAA)

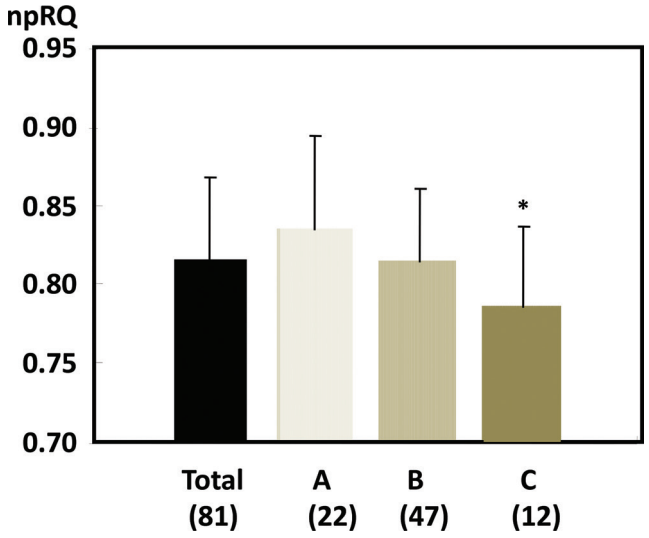


**Figure 3.** Nonprotein respiratory quotients in patients with liver cirrhosis. Eighty-one cirrhotic patients with or without hepatocellular carcinoma who were admitted to Iwate Medical University Hospital were investigated. Energy metabolism was measured by indirect calorimetry (Deltatrac-II Metabolic Monitor, Datax Division Inst. Corp., Helsinki, Finland) in the morning after overnight fasting. npRQ, nonprotein respiratory quotient. Each value is shown as the mean ± standard deviation. *P < 0.05 (compared to grade A). ( ), number of patients with LC.

concentration has recently been reported as an alternative marker to represent npRQ measured by indirect calorimetry to evaluate energy malnutrition in LC [77]. The serum FFA concentration is also a predictor of minimal hepatic encephalopathy diagnosed by computerized neuropsychological testing [78]. Furthermore, our previous study showed that the serum FAA concentration is correlated with the serum acyl-CA to total CA ratio, which would indirectly reflect intracellular mitochondrial function [30]. These findings suggest that the serum FAA concentration in the fasting state may be useful in the assessment of nutritional status in patients with LC.

### 2.4. Glucose intolerance and diabetes mellitus

Glucose intolerance and/or diabetes mellitus is seen in about 30% of patients with LC, though 80% of LC patients have a normal fasting blood glucose level [79]. These manifestations are mainly caused by obesity and increased insulin resistance and hepatitis C virus (HCV) infection. HCV is a major cause of LC and is induced by increased insulin resistance, excess secretion of pancreatic β cells, and portal-systemic shunting [80, 81]. However, insulin resistance improves after eradication of HCV [82]. Age, sex, smoking, excessive alcohol intake, and chronic viral infection (hepatitis B virus and HCV) are established risk factors for HCC [20]. Furthermore, many recent studies have reported that obesity and DM are risk factors for HCC [17–22]. These findings suggest that not only PEM, but also obesity and glucose intolerance or DM might be important factors in the nutritional status that affect the prognosis of LC.

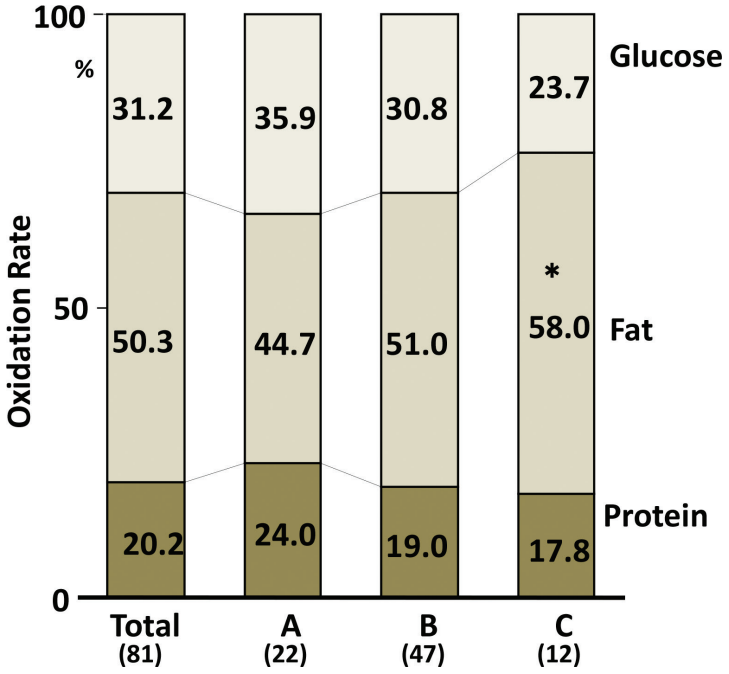


**Figure 4.** Substrate oxidation rates of glucose, fat, and protein using indirect calorimetry in patients with liver cirrhosis. Eighty-one cirrhotic patients with or without hepatocellular carcinoma who were admitted to Iwate Medical University Hospital were investigated. Energy metabolism was measured using indirect calorimetry (Deltatrac-II Metabolic Monitor, Datax Division Inst. Corp., Helsinki, Finland) in the morning after overnight fasting. Each value is shown as the mean. *P < 0.05 (compared to grade A). ( ), number of patients with LC.

## 3. Nutritional management

Based on previous many studies associated with malnutrition including obesity and glucose impairment (DM) in patients with LC, several guidelines on enteral nutrition have been proposed [83–85]. Here, flow chart on nutritional managements for patients with LC shows in **Figure 5**. The recommended dietary managements include energy, protein, fat, sodium chloride, iron, and other nutrient requirement. However, recommended energy intake and protein intake are different between Japan and European Society for parenteral and enteral Nutrition (ESPEN) guidelines (energy intake: 25–35 kcal/kg/day in Japan guideline and 35–40 kcal/kg/day in ESPEN guidelines, and protein intake: 1.0–1.5 g/kg/day in Japan guideline and 1.2–1.5 g/kg/day in ESPEN guidelines). Energy intake should be reduced (25 kcal/kg/day) in patients complicated with DM [85]. Moreover, protein intake involves the protein content of BCAA formulas (BCAA granules or BCAA-enriched nutrient mixture), and it should be reduced to 0.5–0.7 g/kg/day in patients with protein intolerance [85]. Late evening snack (LES) reduces overnight catabolic state in patients with LC

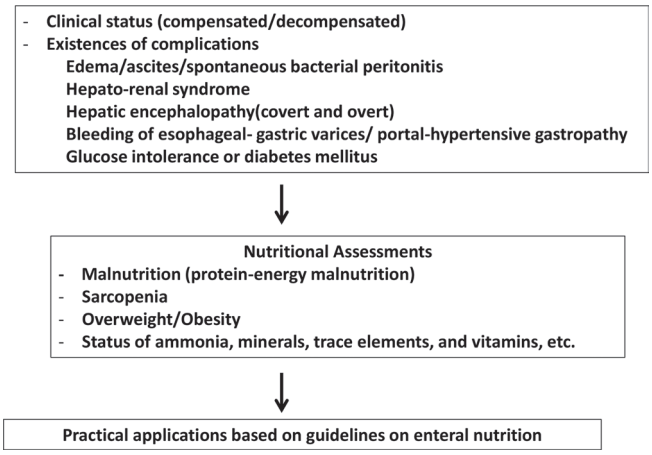


**Figure 5.** Flow chart on nutritional managements for patients with liver cirrhosis.

[86–89]. LES is particularly recommended to the patients with PEM and also useful for managing the blood glucose level in patients with glucose intolerance or DM [90]. As LES, snacks (approximately amounts of 200 kcal) and BCAA-enriched nutrient mixture are usually used. As excess deposition of iron in the liver causes oxidative stress and also promotes hepatocarcinogenesis, so unless severe anemia is observed, an iron-restricted diet 6 mg/kg/day) should be the standard [85, 91]. Zinc supplementation improves the status of hyperammonemia [24–26].

## 4. Conclusion

Nutritional assessment in patients with LC is necessary for the appropriate management of LC patients. PEM, sarcopenia, and obesity are closely associated with adverse outcomes such as liver failure and HCC, as well as graft survival after liver transplantation in patients with LC. However, traditional and newly developed methods of measuring nutritional status are confounded by the changes in metabolism, body composition, and immune function that occur in LC independent of nutritional status. Further studies of precise assessments of malnutrition are needed to improve the prognosis of patients with LC.

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Chapter 8

# Portal Vein Thrombosis in Patients with Liver Cirrhosis

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Additional information is available at the end of the chapter

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**Abstract**

The myth that patients with liver cirrhosis are "auto-anticoagulated" is outdated, and evidence shows that these patients frequently experience thrombosis. Portal vein thrombosis (PVT), although considered as rare, it gradually increases complications that are more likely to occur during late-stage liver cirrhosis. The aim of this chapter is to perform a review of nonmalignant portal vein thrombosis in cirrhosis, in terms of prevalence, pathogenesis, diagnosis, clinical course, and management. Studies were identified by a search strategy using MEDLINE and EMBASE databases. For the MEDLINE search, we used the following terms: ("liver cirrhosis" [MeSH Terms] OR "cirrhosis" [All Fields] OR "cirrhosis" [All Fields]) AND ("portal vein" [MeSH Terms] OR "portal vein" [All Fields]) AND ("Thrombosis" [MeSH Terms]). For the EMBASE search, we used the following terms: (cirrhosis OR phrase liver cirrhosis) AND (phrase thrombosis/OR phrase vein thrombosis/OR phrase thrombosis prevention/OR phrase portal vein thrombosis/ OR phrase liver vein thrombosis/OR phrase mesenteric vein thrombosis/OR thrombosis). Studies were considered eligible if they referred to any aspect of prevalence, pathophysiology, clinical presentation, diagnosis and management, or therapy of PVT in cirrhosis. We put forward possible responses to these unsettled issues starting with prevalence, pathogenesis, and treatment options.

**Keywords:** liver cirrhosis, portal vein thrombosis, treatment

## 1. Introduction

Portal vein thrombosis (PVT) is frequently associated with cirrhosis, mostly in patients with advanced liver disease or hepatocellular carcinoma (HCC). The physiopathology of PVT

development is still under debate, and at the moment, there is a lot of controversy regarding the most efficient treatment. Moreover, the outcome in cirrhotics with PVT awaiting a liver transplant or the influence of thrombosis on posttransplant survival and morbidity is still unknown.

## 2. Epidemiology of portal vein thrombosis in liver cirrhosis

PVT is rarely diagnosed in the general population, the prevalence as reported by autopsy-based studies being up to 1% [1]. Genetic or acquired thrombophilia, mieloproliferative diseases, acute pancreatitis, acute cholecystitis, or other inflammations in the abdominal cavity are the main causes of noncirrhotic PVT [2].

In cirrhosis, PVT prevalence varies between 0.6 and 28% depending on the diagnostic method: imaging exam, during surgery for liver transplantation, or autopsy reports [3–5]. In the last years, PVT prevalence has increased as a result of the widespread use of imaging techniques, such as Doppler ultrasonography, computed tomography, or magnetic resonance, but its exact value is still not known. Studies based on ultrasonography results reported a prevalence of 10–28% in cirrhotic patients, excluding those with HCC [2]. The prevalence of PVT in liver transplant candidates is similar to that in other cirrhotic patients with the same degree of liver disease, although MELD and Child-Pugh scores were higher in patients with PVT, confirming the fact that PVT prevalence increases with the severity of liver cirrhosis. Thus, PVT prevalence is low (1%) in compensated liver cirrhosis and up to 28% in decompensated liver cirrhosis [6–8]. Association between liver cirrhosis and malignancies, especially HCC, may increase PVT prevalence up to 44% [6].

If data on the prevalence of PVT are frequently reported, those on the incidence, however, are quite scanty. Maruyama et al. in a retrospective analysis of 150 patients with cirrhosis, followed up for a median period of 66 months, reported a cumulative overall incidence of PVT of 12.8% at 1 year, 18.6% at 3 years, 20% at 5 years, and 38.7% at 8–10 years [9]. Moreover, the incidence of PVT in patients awaiting liver transplant was reported to be 7% after one-year follow-up [10].

## 3. Pathogenesis of portal vein thrombosis in cirrhosis

Pathogenesis of PVT in patients with cirrhosis still remains uncertain, although some authors consider PVT a complication of liver disease. However, its development is unpredictable and the risk factors are not well recognized. According to Virchow's triad, venous thrombosis is the result of the coexistence of low blood flow, endothelial injury, and a hypercoagulable state. For these reasons, PVT in cirrhosis could be developing as a consequence of portal hypertension, associated with endothelial dysfunction and a relative hypercoagulable state [11, 12].

Portal hypertension is characterized by a reduced portal flow due to increased intrahepatic vascular resistance. This phenomenon is further increased as liver disease progresses [13], representing one of the risk factors that determine the increased incidence of PVT in advanced liver disease as compared to early compensated cirrhosis. This hypothesis was confirmed in one prospective study, which demonstrated that the reduced portal flow velocity below 15 cm/s was the only independent variable correlated with the risk of developing PVT at 1-year follow-up [13].

Advanced cirrhosis is associated with profound and complex coagulation defects, involving procoagulant and anticoagulant factors, fibrinolytic system, and platelets number and function [12]. The net result of all of these defects may be a prothrombotic state, which is likely to be related with the increased endothelial synthesis of von Willebrand factor (vWf) and an increased level of factor VIII, combined with low levels of hepatic anticoagulation agents such as antithrombin III, protein C and S [14, 15].

A number of different inherited and acquired disorders have been also considered as predisposing factors for PVT in patients with cirrhosis, although with variable degree of evidence [16–18]. One study found antiphospholipids antibodies in more than half of cirrhotic patients with PVT [19], whereas variable association of newly recognized risk factors for inherited thrombosis such as the Q506 polymorphism in the gene coding for factor V or the G20210A change in the prothrombin gene (PTHR A20210) has been reported in patients with cirrhosis complicated by PVT [16, 20, 21]. None of these changes were confirmed as independent risk factors for PVT in liver cirrhosis. PAI-1 4G-4G and MTHFR 677TT screening of patients could be useful, especially in alcoholic or cryptogenic cirrhosis, to identify patients in which new drug therapies based on the inhibition of the hepatic stellate cell activation could be easily assessed [22].

Thrombocytopenia was considered for a long time a risk factor for bleeding in patients with liver cirrhosis, but recent reports did not confirm this hypothesis. Some studies showed abnormalities of platelet aggregation in patients with cirrhosis [23, 24], which was attributed to decreased serum levels of clotting factors [23], impaired production of thromboxane A2 and arachidonic acid, or impairment in adhesion molecules [25, 26]. This theory was confirmed by multiple electrode aggregometry, which demonstrated a decreased aggregation activity of platelets, although this phenomenon was not observed under stimulation by ristocetin. This finding implies that the cause of platelet hyporeactivity does not lie in defective transmembrane or postmembrane signaling pathway, while platelet activity was positively correlated with the number of platelets. Interestingly, platelet activity was significantly lower in the PVT group than in the non-PVT group, although the platelet count was not significantly different in either group. A clear reason for this finding was not given, and it is suggested that adaptive changes in platelet function occur after the development of PVT [27]. Some studies consider the degree of thrombocytopenia to be an independent risk factor for PVT, which may seem paradoxical since low platelet count should logically predispose to bleeding. Possibly, as cirrhosis and portal hypertension progress, the resultant decrease in portal flow outweighs a protective effect of low platelet count against thrombosis [27].

Another factor associated with PVT development in liver cirrhosis is endothelial dysfunction. Portal hypertension and inversion of portal vein flow are among the factors associated with endothelial dysfunction. Endotoxemia is another factor that contributes to endothelial dysfunction in cirrhotic patients with PVT. The biological consequences of systemic endotoxemia are low-grade inflammation and peripheral vasodilatation [27]. *In vitro* studies have revealed that lipopolysaccharides, even in low concentrations, may stimulate vWf release from the endothelium [14]. Moreover, Violi et al. provided evidence of a direct correlation between endotoxemia and the ongoing prothrombotic state in the portal venous system [28]. Therefore, it is plausible that endotoxemia, in combination with the coexisting increased vWf release frequently found in cirrhosis, together with portal hypertension may trigger prothrombotic mechanisms, the development of endotoxemia being a surrogate marker of disease severity in patients with cirrhosis [29].

Besides the common risk factors for PVT, other predisposing conditions such as variceal sclerotherapy, liver malignancy, abdominal surgery, or sepsis were described. The roles of sclerotherapy and cyanoacrylate glue injection as potential trigger factors for PVT are controversial, but they were reported in the literature [30]. Such associations could occur as a result of selection bias in patients with more severe portal hypertension. Surgical procedures for portal hypertension were also associated with an increased incidence of PVT [31, 32]. Among them, pericardial devascularization with splenectomy, and splenorenal shunts are associated with an increased risk of PVT [33].

Along with the sluggish portal flow [19] and the presence of liver malignancies (i.e., hepatocellular carcinoma), other acquired local (abdominal surgery, trauma or bacterial infection, and portacaval shunts), or general (sepsis and myeloproliferative disorders) factors have been claimed as possible causes of PVT in patients with liver cirrhosis [12–16].

The main consequences of PVT are related to the extension of the thrombus and include intestinal ischemia and acute/chronic portal hypertension. Gastrointestinal bleeding due to portal hypertension following PVT has been reported as a major cause of death in patients with cirrhosis [34]. The pathogenesis of PVT in such patients remains unclear, although decreased portal vein blood flow, a hypercoagulable state, and systemic inflammation may be of importance. Despite the great number of risk factors for PVT in liver cirrhosis, thrombosis itself should be considered a multifactorial disease, and the likelihood of developing PVT increases in direct proportion to the number of risk factors present in each patient.

## 4. Diagnosis of portal vein thrombosis

PVT diagnosis in cirrhotic patients involves clinical suspicion with further imagistic confirmation. According to the moment of diagnosis, this particular type of venous thrombosis could be classified as:

- *acute*: sudden formation of a thrombus within the portal vein, with or without involvement of the mesenteric and/or splenic vein [35];
- *chronic:* the obstructed portal vein is replaced by collateral veins bypassing the thrombosed vein [36].

### 4.1. Clinical presentation

PVT is frequently diagnosed in asymptomatic cirrhotic patients by routine abdominal ultrasound (US). In most of these cases, PVT is chronic with partial obstruction. Acute partial or total PVT is frequently symptomatic, and it is associated with decompensation or further decompensation of liver disease.

The symptoms and signs of acute PVT could be represented by severe abdominal or lumbar pain with sudden onset, progressive over days, without peritoneal signs when the superior mesenteric vein is involved, functional ileus, ascites, or variceal bleeding. The majority of the patients with acute PVT associate systemic inflammatory response syndrome in the absence of sepsis. If the symptoms are not resolved in 5–7 days or liver cirrhosis is complicated by further decompensation and clinical deterioration, mesenteric vein involvement with complete loss of blood flow should be suspected.

Chronic PVT is asymptomatic in most cases. The pain is a sign of mesenteric vein thrombosis and bowel ischemia. Although there is a minimal change in the hepatic arterial blood supply, the portal pressure is increased, with the development of portosystemic collaterals and an increased risk of variceal bleeding. This fact supports the Baveno VI recommendations stating that it is mandatory to perform screening endoscopy in all patients diagnosed with chronic PVT within 6 months from the acute episode if a complete recanalization of thrombosis is not achieved [36]. A total of 22% of patients without varices at initial endoscopy will develop this condition in 3 years [37]. Therefore, a follow-up endoscopy should be performed in subjects without varices at the baseline [36].

With regard to primary prevention of bleeding, no randomized controlled trial compared the effectiveness of nonselective beta-blockers versus endoscopic band ligation in PVT. In this scenario, as well as in the context of the acute bleeding and secondary prophylaxis, Baveno VI recommends following the guidelines on PH in cirrhosis [36]. Besides prehepatic portal hypertension, portal cholangiopathy is another context associated with chronic PVT. Patients develop jaundice, abdominal pain, and episodes of cholangitis.

### 4.2. Imaging evaluation: abdominal ultrasound

When PVT is suspected, ultrasound is the first-line imaging method to be used, since it holds an accuracy ranging from 88 to 98% for the detection of PVT with a sensitivity and specificity of 80–100% in the majority of studies [38, 39]. The sensitivity of ultrasound is particularly high in complete PVT, while the risk of false-negative results occurs only in incomplete PVT [40] and isolated superior mesenteric vein thrombosis [38]. In two-dimensional (2-D) Gray-Scale ultrasonography, a thrombus appears as a hypo/isoechoic material occupying part of (partial thrombosis) or the entire vessel (complete thrombosis). The normal portal vein can be eventually replaced by multiple tortuous vessels with hepatopetal flow, a condition named as "cavernomatous transformation" or "cavernoma," easily detected with Doppler ultrasound. Color/power and pulsed Doppler should be mandatorily used to confirm whether the vessel has a remnant blood flow, to help differentiate high-degree partial thrombosis from complete thrombosis. The reliability of ultrasonography in the detection of PVT improves with the operator's experience, and whenever PVT is clinically suspected, ultrasonography

should be performed by experienced operators [41]. Ultrasonography suffers from other limitations such as reduced visualization in obese individuals and in case of abundant bowel gas, and impossibility to assess bowel ischemia. This should be suspected in case of ascites and/or high blood lactate levels. Ultrasound is sufficient to diagnose PVT in patients with a good acoustic window, but when ultrasonography is insufficient, a second-line cross-sectional imaging method should be considered to confirm or exclude the diagnosis.

### 4.3. Imaging evaluation: computed tomography and magnetic resonance

Contrast-enhanced four phase (pre-contrast, arterial, portal, and late) CT (CECT) and contrast-enhanced MRI (CEMRI) can be used, with CT is preferred in unstable patients with acute abdominal symptoms. Advantages of MR and CT over US include the possibility of detecting bowel ischemia, septic foci and intraabdominal malignancies, and higher sensitivity in the detection of thrombosis in the splenic and superior mesenteric vein. Among the well-known drawbacks of CT are exposure to ionizing radiation, the risk of allergic reactions, and nephrotoxicity. CEMRI is also contraindicated in patients with acute renal failure because of the risk of nephrogenic systemic fibrosis. Once PVT is diagnosed, CECT or CEMRI is mandatory to evaluate the extent of thrombosis and to allow a detailed mapping of portosystemic collaterals, crucial to the planning of interventions aimed at recanalizing the portal venous system. It should be considered that clinical consequences of PVT mainly depend on the number of vessels completely occluded [42], as well as the degree of collateralization in chronic cases. Furthermore, the presence of ascites is a predictor of the lack of response to anticoagulation and should be reported [42]. Several classification/staging systems have been developed, but they rely heavily on anatomical considerations. The most commonly cited and used in clinical trials is the one proposed by Yerdel et al. [43]. However, there is no validated classification to be used in clinical practice in order to personalize risk assessment and guide therapy [44].

Both Doppler ultrasonography and multiphasic-computed tomography have high sensitivity and specificity for PVT detection [45]. Doppler US is highly accurate in detecting thrombosis involving the trunk of the portal vein and intrahepatic branches, also providing additional information regarding the portal flow and its direction. CT is better at assessing the superior mesenteric vein, spontaneous portosystemic shunts, renal veins, and the inferior vena cava. While a CT exam is generally performed at the time of initial evaluation for liver transplant, Doppler ultrasound is appropriate for follow-up imaging as it can be performed repetitively and does not have the risks of intravenous iodine contrast and radiation.

### 4.4. Imaging evaluation: malignant versus nonmalignant PVT

Patients with cirrhosis or neoplastic disease may develop either benign or malignant PVT. In patients with HCC, it is essential to radiologically distinguish tumor invasion of the main trunk or the branches of the portal vein as the cause for PVT versus bland thrombus in the portal vein because this could determine the proper therapeutic approach and their prognosis.

This is not without implications since the major vascular tumoral invasion is an absolute contraindication to transplant, while bland PVT in the presence of HCC needs to be approached similarly to a non-HCC setting [45]. Tumor-related PVT is usually detected in portal vein branches adjacent to and in direct continuity of the tumor, and is often associated with a high alpha-fetoprotein level.

Until recently, imaging differentiation of the benign from the malignant PVT has depended on the findings of contrast enhancement and luminal expansion on abdominal ultrasound, CT, or MRI. Signs of malignant PVT on ultrasound include an expansive aspect mass inside the lumen, with heterogeneous aspect and disruption of portal vein walls. Color/power-Doppler ultrasound shows signs of neovascularization within the mass, and pulsed Doppler could confirm arterial flow with a high resistance index associated with malignant PVT. One of the most sensitive and with small additionally methods for malignant PVT diagnosis is contrast ultrasound. In contrast to bland PVT, which remains unenhanced in all phases, a malignant PVT shows the same contrast-behavior as HCC—rapid wash-out (hypoperfusion in comparison to the rest of the liver parenchyma) in the portal/late phase.

Enhancement or an increase in density or intensity on CT or MRI, respectively, after contrast administration could also establish the diagnosis of malignant PVT. Conversely, absent enhancement confirms bland thrombus.

Careful screening for PVT is important in all patients with cirrhosis and in those under evaluation for liver transplantation. Repeated imaging at specified intervals—usually every 3 months, during the pretransplant waiting period—is also recommended in order to detect thrombosis that may develop during follow-up [7]. Patients who develop unexplained worsening of liver functions or gastrointestinal bleeding despite adequate prophylaxis should also be evaluated for PVT of recent onset.

## 5. Management of portal vein thrombosis

Nowadays, there are two main possibilities of PVT treatment: anticoagulation with low-molecular-weight heparin (LMWH) or oral anticoagulants, and transjugular intrahepatic portosystemic shunt (TIPS). The best therapeutic solution is still under debate, but the final goal is to prevent PVT extension to the mesenteric veins and achieve PVT recanalization (**Figure 1**).

### 5.1. Anticoagulant treatment for PVT in cirrhotic patients

Anticoagulant treatment in cirrhotic patients who are not on a liver transplant list may be considered if the superior mesenteric vein is involved or the patient carries a known prothrombotic condition [36].

Some studies have reported that spontaneous recanalization of the portal vein in the absence of an anticoagulant treatment is unusual. In the study by Francoz et al., no patient achieved recanalization in the absence of anticoagulation, while 42% achieved recanalization while

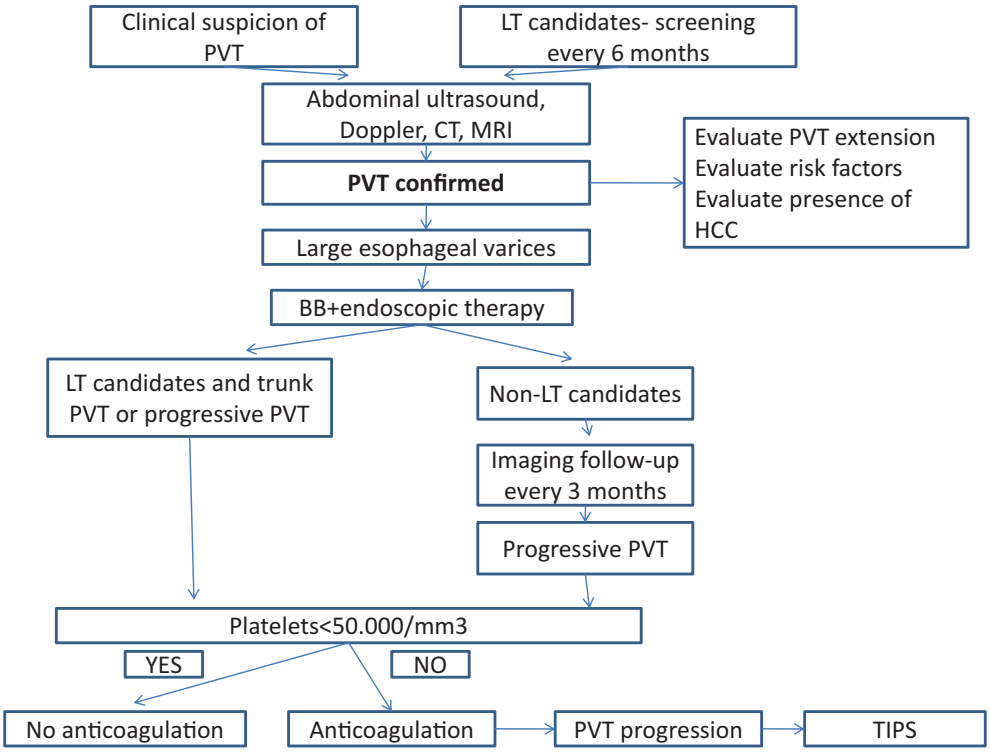


**Figure 1.** Algorithm for the diagnosis and management of PVT in liver cirrhosis.

under anticoagulant therapy [46]. Senzolo et al. reported thrombus progression in 75% of patients who did not receive anticoagulant treatment, compared to only 15% of treated patients [47].

There are limited studies reporting on the use of anticoagulation for PVT in patients with cirrhosis. In all these studies, complete recanalization has been described in 33–45% of cases, while partial portal vein recanalization was observed in 15–35% of cases [46, 48, 49]. In a study by Senzolo et al., prospectively enrolling 56 individuals (35 treated and 21 controls), complete recanalization was achieved in 36% of subjects and partial recanalization in 27%, after therapy with LMWH (mean 5.5 months) [47]. The time between diagnosis and anticoagulation—under 6 months—was the most important factor positively associated with portal vein recanalization. In a study from Spain, by Delgado et al., including 55 cirrhotic patients, the majority of them (75%) diagnosed with partial PVT, complete portal vein recanalization was achieved in 45% of cases after a median duration of therapy of 6.3 months with vitamin K antagonists (VKA) or LMWH [48]. In this study, the only predictive factor for achieving complete portal vein recanalization was also early initiation of anticoagulation therapy after diagnosis, in less than 14 days.

Nowadays, there are no clear data regarding the duration of anticoagulant treatment, although Amitrano et al. treated 28 patients with LMWH and demonstrated that after 6 months, complete portal vein recanalization was achieved in 33% of cases and partial portal vein recanalization was observed in 50%. In individuals with partial response to therapy, anticoagulant treatment was continued for more than 6 months, and 86% of these patients achieved complete recanalization [50].

The rate of PVT recanalization depends not only on the time of PVT diagnosis, but also on the type of PVT in most of the cases: complete or partial, tumoral or nontumoral. As shown by most studies, recanalization is uncommon in patients with complete thrombosis, but anticoagulation is still indicated in order to prevent the extension of the thrombus [46–50]. However, it is unclear what proportion of these patients would have recanalized spontaneously and, more importantly, whether they derived any clinical benefit from anticoagulation. This hypothesis was raised by other studies with conflicting results. Maruyama et al. reported a spontaneous improvement in 47.6%, unchanged appearance in 45.2%, and progression in only 7.2%. There was no significant difference in the natural course of thrombosis, based on the degree of obstruction or the location of the thrombus, and recurrence of PVT after spontaneous resolution was observed in 21.4% [9]. Our data also confirmed Maruyama's study results. We demonstrated that in most of the cirrhotic patients diagnosed with PVT, the thrombus remained with the same dimensions or disappeared without any therapeutical intervention [51].

For cirrhotic patients diagnosed with PVT awaiting for a liver transplant, it is important to achieve recanalization and thus achieve a physiological portal vein anastomosis in order to ensure portal flow to the graft. Transplanting patients with PVT extended to the superior mesenteric vein or with extensive portal vein thrombosis is associated with higher morbidity and mortality, PVT being a predictor of posttransplant mortality in some studies [43, 52, 53].

An important objective in the management of PVT in cirrhotic patients awaiting liver transplantation is to achieve recanalization for the end-to-end portal vein anastomosis to be surgically possible. Another objective is to prevent extension of the thrombus to the splenic and superior mesenteric vein, since these veins can also be used to restore portal flow to the graft in case the main portal vein is thrombosed. In the event that neither the portal vein nor the superior mesenteric vein can be used, nonanatomical techniques to restore portal flow are possible, but these are associated with increased morbidity and mortality. Francoz et al. compared 19 individuals with cirrhosis and PVT on the waiting list for liver transplantation who received anticoagulation therapy (VKA) with 10 individuals not receiving therapy. A total of 42% of treated individuals achieved complete PV recanalization. None of the untreated patients had recanalization, and, in fact, PVT progressed in 60 % in the untreated group. Moreover, anticoagulation therapy did not increase blood loss during liver transplantation [46].

The rationale for treating PVT in patients with cirrhosis is that it increases morbidity compared to matched cirrhotics without PVT, although there is controversy regarding the influence of PVT on the natural course of liver cirrhosis. PVT has been reported to be independently associated with a higher risk of failure in controlling acute variceal bleeding as well as rebleeding [44]. The occurrence of PVT has also been shown to increase mortality, which has been observed even

in patients with lower Child-Pugh scores [46]. Recanalization of PVT has also been reported to reduce esophageal variceal pressure, improving morbidity, and mortality rates [44].

There are no clear recommendations for an optimal anticoagulation regimen for the treatment of PVT in patients with cirrhosis. Monitoring of anticoagulation regimen is complex in the cirrhotic patient and, therefore, choosing between different anticoagulants (LMWH, VKA, or the new oral anticoagulants) is a difficult decision. LMWH is less practical for patients, since it necessitates daily subcutaneous injections, although it does not affect INR values and, consequently, does not interfere with MELD or Child scoring. There is, however, limited information on the pharmacodynamic profile of LMWH in cirrhotic individuals.

Cirrhotic patients often have an increased volume of distribution because of fluid overload, and this makes it difficult to determine the optimal dose of LMWH. Moreover, the major route of *elimination* of the *LMWH* is through the *kidneys*, and, since many patients with cirrhosis have renal insufficiency, the half-life of LMWH is increased. The only method of LMWH treatment monitoring validated until known is by determining the anti-Xa activity, but this method is unreliable in cirrhosis [35, 55].

The primary problem with VKA is determining the adequate anticoagulation in patient with cirrhosis who already has an altered abnormal prothrombin time. Most studies have targeted an INR of 2–3 [54]. Based on an empirical experience not relying on randomized studies, if the baseline INR is over 2, it is difficult to determine if a given dose of VKA ensures adequate anticoagulation. It may also be difficult to determine the optimal INR target for dose adjustment. There is also a potential risk of further lowering of protein C levels with the use of VKA, and this could theoretically increase the prothrombotic imbalance of individuals with cirrhosis.

The new oral anticoagulants—thrombin inhibitors and inhibitors of activated factor X such as dabigatran and rivaroxaban—offer the advantage of oral administration, the absence of laboratory monitoring, and an antithrombin-independent mechanism of action [54]. However, there are a few reports regarding their use in cirrhotic patients, most of them isolated cases. One of the major disadvantages of these new anticoagulants was the absence of an antidote. This problem was solved for dabigratan and also for rivaroxaban, which could be the new class of anticoagulants preferred in PVT treatment. In cirrhotic patients, it may be necessary to reverse anticoagulation during episodes of inadvertent bleeding or at the time of surgery. While the effect of VKA can be expertly reversed by fresh-frozen plasma or prothrombin complex concentrate, there is no potent and rapidly acting antidote to reverse the effect of LMWH or the newer thrombin inhibitors.

Even if the anticoagulant treatment seems to be the same in patients with liver cirrhosis, it is uncertain whether it is beneficial to anticoagulate asymptomatic patients who are detected with PVT incidentally on imaging [35, 55].

The impact of PVT on the natural history of cirrhosis remains a matter of great debate, and the clinical benefits of PV recanalization have fully demonstrated [50]. Despite this, there is evidence that cirrhotic individuals with PVT awaiting for liver transplantation should be treated with anticoagulation therapy because complete or partial portal vein recanalization has been associated with a better 2-year survival rate after liver transplantation (82–83%) compared to

individuals with complete PVT (50%) [46]. Other situations where anticoagulation is expected to be beneficial are cirrhotic patients with acute PVT with extension to the superior mesenteric vein [35, 55]. Cirrhotic patients with well-documented prothrombotic disorder should obviously be considered for anticoagulation. Patients with cavernomatous transformation of the portal vein have been excluded from most trials since such patients are not expected to benefit from anticoagulation.

### 5.2. TIPS and thrombolysis for PVT in cirrhotic patients

The use of transjugular intrahepatic portosystemic shunt (TIPS) has also been reported to recanalize the portal vein and also prevent rethrombosis by restoring portal flow through the shunt [56–59]. TIPS insertion and recanalization is associated with mechanical thrombectomy. However, in such cases, TIPS is expected to be technically challenging with a higher failure rate and should be attempted only in experienced centers. Systemic or *in situ* thrombolysis has been reported in cirrhotic patients with PVT [60]. In noncirrhotic patients with acute PVT, rates of recanalization have been dismal with attempted thrombolysis. There has also been a high incidence of major bleeding [60]. There are no data to support this option in this setting. TIPS promotes the dissolution or decrease in PVT, splenic, or mesenteric veins, in the US population of patients with predominantly compensated liver cirrhosis of various etiologies [57, 58].

## 6. Portal vein thrombosis and liver transplantation

Most of the studies on liver transplant patients with PVT revealed higher technical difficulties and mortality, postoperative complications, in the PVT group compared with those without PVT. The higher morbidity and mortality is multifactorial and is related to a more complex surgical procedure, increased requirement of blood transfusions, higher risk of complications such as primary nonfunction or dysfunction, hepatic artery thrombosis, postoperative pancreatitis, sepsis, or renal failure [61, 62]. Moreover, there is a high risk of 9–42% of PVT rethrombosis [63]. Patients with Child-Pugh class C cirrhosis, complete PVT, and alcoholic etiology of hepatic disease have a higher risk of PVT rethrombosis after liver transplant. Of a pooled total of 169 patients with partial PVT, 7 (4%) developed rethrombosis in contrast with 14 of 114 patients with complete PVT (12.3%) [63].

The main treatment indication is early anticoagulation with low-molecular-weight heparin unless it is contraindicated for surgical reasons, although randomized controlled trials are lacking. Moreover, there is no consensus on how long anticoagulation should be continued posttransplant. In the absence of prothrombotic state, there is no evidence that pretransplant PVT justifies long-term anticoagulation posttransplantation. Mortality is related to the grade of preoperative PVT. The 30-day mortality in patients undergoing liver transplantation with or without PVT has been reported as 10.5% versus 7.7%, respectively [63]. The 1-year mortality was also reported to be significantly higher in a systematic review according to the presence (18.8%) or absence (15.3%) of PVT [63]. The 30-day mortality has been reported to vary between 3.8% for grade 1 and 2 PVT, and going up to 27% for grade 4 PVT [64]. Preoperative

PVT seems to influence early outcome more than long-term results, with the maximum decrease in survival occurring in the first year, and medium-term results with or without PVT appearing to be comparable if early mortality is excluded [65].

For many years, PVT had been considered as an absolute contraindication to liver transplantation [66]. The first successful surgery for complete PVT was reported by Shaw et al. in 1985 [66]. Nowadays, the innovations in surgical techniques have made it possible to overcome problems due to PVT during transplantation. The stage of liver disease and the collateral circulation increase the complexity of surgical techniques and pose a challenge for the surgery, because it is very important to have an adequate portal inflow of the graft to maintain the liver function.

In order to establish if the patient has a surgical indication, preoperative assessment must evaluate the correct stage and grade of PVT based on a spiral CT scan or a magnetic resonance venogram. For surgical purposes, Yerdel et al. have classified PVT into four grades [43]:

Grade 1: Partially thrombosed portal vein, where the thrombus occupies less than 50% of the lumen.

Grade 2: More than 50% occlusion of the portal vein, including total occlusions, with or without extension into the superior mesenteric vein.

Grade 3: Complete thrombosis of both the portal vein and the proximal superior mesenteric vein.

Grade 4: Complete thrombosis of the portal vein, proximal, and distal superior mesenteric vein.

There are several available surgical techniques for PVT reconstruction during liver transplant surgery. All the techniques vary according to the degree and the anatomical spread of the PVT [65].

1. Portal vein thrombectomy (for Yerdel grade 1 and 2 PVT) and direct anastomosis of donor and recipient portal vein. A recent study suggested that 75–90% of transplants performed in patients with PVT, and the thrombosis could be managed only by thrombectomy [61]. After completion of the thrombectomy, adequate flow in the recipient portal vein or superior mesenteric vein must be confirmed by releasing the vascular clamp before proceeding with the anastomosis.
2. In cases of Yerdel grade 2 or grade 3 occlusions, an anastomosis may be required between the graft portal vein and the recipient superior mesenteric vein. The anastomosis uses a section of the donor iliac vein as a graft. The presence of a large collateral vein may provide an alternative portal inflow, although extraanatomical vessels are more fragile and prone to thrombosis.
3. Arterialization of the portal vein: anastomosis of the graft portal vein to the recipient arterial inflow.
4. Portacaval hemitransposition: an anastomosis of the graft portal vein is made to the suprarenal recipient inferior vena cava. The disadvantage of classic portacaval hemitransposition

is the persistence of portal hypertension associated with an increased risk of bleeding from gastroesophageal varices, which may occur in up to 50% of such cases [65].

Rodriguez-Castro, in a systematic review, reported that among 49 patients with portacaval hemitransposition, 20% had episodes of variceal bleeding, 58% had persistent ascites, and 26% presented with renal dysfunction after liver transplantation [66]. An alternative to portacaval hemitransposition is renoportal transposition, where the recipient portal vein is anastomosed to the left renal vein [65].

## 7. Conclusion

PVT is a highly heterogeneous entity regarding its underlying risk factors and the association with liver cirrhosis independently of the disease stage. Although significant advances have been made in the field of PVT associated with liver cirrhosis in recent years, many important questions still remain unanswered. Most critical issue that requires future studies is the influence of PVT on natural course of liver cirrhosis according to the new classification, and it has to establish the risk-benefit ratio of anticoagulant treatment in different groups of patients, including the role of the new oral anticoagulant.

## Abbreviations

| | |
|---|---|
| PVT | Portal vein thrombosis |
| LT | Liver transplant |
| CT | Computed tomography |
| MRI | Magnetic resonance imaging |
| BB | Beta-blockers |
| TIPS | Transjugular portosystemic shunt |
| HCC | Hepatocellular carcinoma |

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Chapter 9

# Hemodynamic Optimization Strategies in Anesthesia Care for Liver Transplantation

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Additional information is available at the end of the chapter

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**Abstract**

In this chapter, aspects of hemodynamic regulation in the end-stage liver disease (ESLD) patient, factors, contributing to the hemodynamic profile, coagulation-related problems, blood products transfusion tactics and problems, and hemodynamic optimization strategies during different stages of liver transplantation procedure—specifically what, when, and how to correct, with special attention to vasoactive agents use, will be discussed.

**Keywords:** liver transplantation, anesthesia, hemodynamic optimization, vasoactive agents, transfusion management

## 1. Introduction

Inseparable part of liver transplantation procedure, anesthesia, and perioperative care for the liver transplant recipient has made a remarkable progress during last decades, becoming a clinical specialty with well-defined goals, requirements, and approaches. Today, with a rapid expansion of liver transplant programs worldwide and growing numbers of liver transplant procedures performed, many aspects of anesthesia care, complicated and risky in the relatively recent past, have become routine and safe. And yet some problems remain unresolved, still posing a challenge for anesthesiologist in the field. Despite incessant and plentiful research, investigating literally every imaginable aspect and angle of the anesthesia and perioperative care for liver transplant recipient, and myriad of publications coming out every year, no consensus has been reached so far as for the best choice of anesthesia induction and maintenance, intraoperative hemodynamics management, fluid and blood products transfusion, patient's monitoring, and more. One of the most important time- and effort-consuming

aspects of anesthesia care, expanding well beyond proper intraoperative time onto the first long hours of ICU stay, is patient's hemodynamic management. Its multicomponent nature, sometimes a very short time resolution in the decision-making process, poorly predictable course of patients reactions, overall instability with rapid, oftentimes detrimental and life-threatening changes makes management of patient's hemodynamics an extremely challenging and complicating task.

## 2. Factors contributing to hemodynamic profile of the ESLD patient

Typical hemodynamic pattern of end-stage liver disease (ESLD) patients includes high cardiac output (CO)/cardiac index (CI)—hyperdynamic circulation pattern, with normal-to-low mean blood pressure, variable central venous pressure (CVP), along with general arterial and venous vasodilatation due to substantially decreased systemic vascular resistance (SVR). The hyperdynamic circulation is thought to be a compensatory change, induced by splanchnic and peripheral vasodilatation, reducing the effective blood volume. This, and also decreased perfusion pressures, leads to a diminished renal blood flow in cirrhotic patients, which in turn stimulates the renin-angiotensin-aldosterone system and antidiuretic hormone production, resulting in renal artery vasoconstriction, sodium retention, and volume expansion. Worsening liver disease results in progressive vasodilatation, making the hyperdynamic circulation and renal artery vasoconstriction more pronounced [1].

Arterial vascular tone is regulated by complex interactions of different vasoactive substances, namely catecholamines and NO complex. In ESLD patients, sensitivity of β-adrenoreceptors is relatively decreased, causing cardiovascular response to endogenic catecholamines substantially attenuated [2]. Plasma-free norepinephrine and epinephrine levels are significantly higher. Fraction of epinephrine, contributing to total catecholamines, increased up to 50% (normal: about 17%). Dopamine concentration is unchanged [3].

In recent years, nitric oxide (NO) has been recognized as the most important vasodilator of the splanchnic and systemic circulation. Cytokines, especially TNF-$\alpha$, are considered to be NO inducers. Endothelial NO synthase has been found as a main source of the vascular NO overproduction in the splanchnic arterial circulation [4–6].

Augmented intrahepatic vascular resistance due to sinusoidal constriction is considered the major cause of portal hypertension. Hepatic stellate cells (HSC) provide a basis for control of sinusoidal vascular tone and an arrangement for sinusoidal constriction and hepatic blood flow (HBF) reduction. The dynamic part of hepatic resistance is caused by active contraction/relaxation of HSC. Portocaval collaterals divert up to 80% of blood flow away from liver [7].

Cardiomyopathy plays a substantial role in the hemodynamic profile and cardiovascular compensation mechanisms in a cirrhotic patient. The characteristic features of cirrhotic cardiomyopathy include an attenuated systolic or diastolic response to stress stimuli, structural and histological changes of myocardium, electrophysiological abnormalities, and increased concentrations of serum markers, suggestive of cardiac stress. The impaired cardiovascular

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responsiveness in cirrhosis is likely related to a combination of factors that include among other reasons, β-adrenergic receptor dysfunction and reduction of β-adrenergic receptor density in cirrhotic patients. Recently, it has been found that, in cirrhotic patients, the control of vascular tone by $Ca^{++}$ and $K^{+}$ channels is altered. The calcium channel dysfunction, leading to decreased cardiomyocyte contractility, was demonstrated in an animal model study [2, 8–10].

Albeit commonly overlooked, many of these pathogenic mechanisms resulted in RV overload with gradual dilatation and impaired contractile function, leading to elevated mean pulmonary artery pressure (MPAP). Despite characteristically increased resting CO, ventricular contractile response is, actually, substantially attenuated. Cardiomyopathy may contribute to portopulmonary hypertension.

However, overt severe Congestive Heart Failure (CHF) is rare. Increased intra-abdominal pressure (ascites) contributes to both portal and PA hypertension [11].

Pulmonary vascular changes in cirrhosis are often quite substantial. They include portopulmonary hypertension (POPH) syndrome, which entails development of pulmonary hypertension in a cirrhotic patient with portal hypertension, and also hepatopulmonary syndrome, which is, essentially, increased pathological shunting and V/Q mismatch due to development of the arteriovenous malformations in the lung, resulting in hypoxemia. Portopulmonary hypertension is less prevalent than hepatopulmonary syndrome with an estimated prevalence of about 5%.

POPH is best defined as pulmonary arterial hypertension (PAH). Necessary conditions include presence of portal hypertension and absence of other secondary causes of PH, such as valvular disease, chronic thromboembolism, collagen vascular disease, or exposure to certain drugs or toxins. Current diagnostic criteria include the presence of portal hypertension (either inferred from the presence of splenomegaly, thrombocytopenia, portosystemic shunts, esophageal varices or portal vein abnormalities, or confirmed by hemodynamic measurements), but not necessarily the presence of cirrhosis; and hemodynamic parameters, specifically MPAP >25 mmHg at rest, >30 mmHg with exercise/stress, PCWP<15 mmHg, PVR>120 dynes/s/$cm^5$, and transpulmonary gradient >10 mmHg [12–16].

A most common suggested mechanism for POPH maintains that the increased blood flow (high cardiac output) in chronic liver disease causes pulmonary vascular wall shear stress, which can trigger the dysregulation of numerous vasoactive substances. The presence of portosystemic shunts may lead to the shunting of vasoactive substances from the splanchnic to the pulmonary circulation, causing deleterious effects in the pulmonary vasculature [17, 18].

The severity of hepatopulmonary syndrome is classified according to the degree of arterial hypoxemia, specifically mild ($PaO_2$ of 60–80 mm Hg), moderate (50–60 mm Hg), and severe (<50 mm Hg). Intrapulmonary vascular dilation leads to increased V/Q mismatching plus a degree of intrapulmonary shunting of deoxygenated, mixed venous blood. Both these mechanisms cause systemic arterial hypoxemia [19–22]. Impairment of hypoxic pulmonary vasoconstriction means that gravitational effects on pulmonary blood flow are poorly tolerated. Many authors observed at least partial resolution of the hepatopulmonary syndrome following liver transplant [23, 24].

A common complication of liver disease and portal hypertension is the accumulation of ascites, whereas the presence of significant ascites sometimes compromises respiratory function mostly by creating the restrictive pattern of lung mechanics, a more significant complication is the presence of fluid in the thorax, termed hepatic hydrothorax. Hydrothorax may exacerbate the restriction pattern even further, sometimes leading to atelectasis development, with associated V/Q mismatch and intrapulmonary shunt that adds to already pre-existing hypoxemia, and also to increase of PA pressure.

## 3. Hemodynamic changes during orthotopic liver transplant surgery

### 3.1. Anesthesia-related factors

From the days, when the first successful liver transplantation surgery was performed to this day, anesthesiologists all over the world, despite plenty of ongoing and already published research works in the field, have not yet arrived at a consensus, let alone adopted unified guidelines or protocols of the anesthetic technique for liver transplantation surgery.

Since anesthesia-related systemic hemodynamic changes are well described elsewhere, the only aspect of these effects, specifically an impact of anesthesia factors and adjuvant drugs on hepatic blood flow (HBF) and oxygen delivery, needs to be discussed here. The degree to which the hemodynamic changes, caused by anesthetic agents, take place in patients with advanced liver disease, depends on the patient's particular hemodynamics, volume status and compensation pattern, nature of the surgical procedure, and many other factors. Patients with cirrhosis may be more sensitive to hepatic hypoperfusion, and may be more susceptible to liver injury (such as administration of a hepatotoxic drug, rapid blood loss).

It has been shown that practically all general anesthesia techniques, regardless of drug combinations, in the absence of surgical stimulation, reduce the HBF by about 30%. It appears that the systemic arterial blood pressure is a main determinant of hepatic blood as the hepatic artery exhibits almost no autoregulatory capacity [25]. Commonly used IV induction anesthetic agent, etomidate, along with maintaining well the systemic hemodynamic parameters at baseline levels, only moderately reduces the HBF in a dose-dependent manner, and causes the increase in hepatic arterial resistance (by 40%).

Propofol, however, has shown an ability to preserve baseline levels of the HBF, as long as systemic hemodynamic changes were insignificant [26].

Use of isoflurane and sevoflurane for anesthesia maintenance, albeit being associated with minimal-to-moderate global reduction of HBF, has not been found to be associated with any significant influence on arterial hepatic blood flow or oxygen transport and extraction ratio in the liver. Short-action opioids, fentanyl in particular, has shown no discernible effect on HBF [27–31].

Other potential perioperative causes of a reduction of HBF include mechanical ventilation, positive end-expiratory pressure, systemic hypotension due to hypovolemia, hemorrhage, etc., and hypoxemia. Beta ($\beta$)-blockers, alpha ($\alpha$)-agonists, $H_2$ blockers, hypocapnia, alkalosis, and hypoglycemia have been found to be associated with moderate HBF reduction.

Dopamine (3 mcg/kg/min), epinephrine (from 0.01 mcg/kg/min), hypercapnia, acidosis, and hypoxemia, however, are among the factors that actually can increase HBF [32, 33].

With a substantial variety of anesthetic techniques currently in use and with full awareness of ESLD hemodynamic profile specifics and patient-to-patient variety in that respect, it appears to be reasonable to set hemodynamic goals (i.e., hemodynamic parameters to possibly maintain) for anesthesia care for liver transplant. These should include mean arterial pressure (MAP) around 75–85 mmHg, Heart rate (HR): <100/min, Central venous pressure (CVP): <20 mmHg, Mean Pulmonary Artery Pressure (MPAP): <25 mmHg, CO/CI: >4 L/min/>2 L/min $m^2$, Systemic Vascular Resistance (SVR): >500 dynes/s/$cm^{-5}$, and mixed venous SvO2: >75%.

### 3.2. Surgery-related factors

The course of liver transplantation surgery includes four stages. During preanhepatic, or dissection phase, the diseased liver is being dissected and prepared for removal. Portal vein clamping, followed by hepatic artery and IVC clamp, heralds the start of anhepatic phase, during which part of the diseased liver is being removed from the body and being replaced by the donor's organ. Vascular anastomoses are being performed, followed by organ reperfusion phase, the shortest one with most significant hemodynamic impact. After venous blood flow restoration in the transplanted organ, postreperfusion phase include common hepatic arterial anastomosis, cholecyctectomy, and bile duct reconstruction.

During preanhepatic (dissection) phase, laparotomy, often followed by ascites evacuation, causes drop of intra-abdominal pressure, with rapid splanchnic volume increase (i.e., mesenteric blood pooling) ensued. Ongoing blood loss at this stage may be very substantial, due to abundance of venous collaterals in cases with longstanding portal hypertension, and also in cases of re-do transplants, or cases with significant adhesions after previous surgeries. Decrease of venous return, ongoing blood loss, fluid shift, and developing acidosis further contribute to CO/CI and mean arterial blood pressure (MABP) decrease.

Portal cross clamp, which portends the anhepatic stage start, causes variable (20–30% of baseline) degree of venous return decrease. However, in cases of well-developed portocaval collaterals (longstanding portal hypertension), this loss of preclamp venous return may be less significant, around 15–20%, and generally well tolerated. IVC complete cross-clamp oftentimes leads to a more substantial and poorly tolerated (approximately 50%) decrease of venous return, whereas IVC partial clamp causes variable, about 25–50%, decrease of venous return [34, 35]. ESLD patients have very limited ability, if any, to compensate for the rapid decrease in venous return with systemic vasoconstriction due to inherent low SVR. Veno-venous bypass (VVB) may present a possible solution to compensate for decreased venous return. Hemodynamic instability following test clamping of IVC is the most common indication for initiating VVB [36]. It has been suggested [37] that hypotension (30% decrease in MAP) or a decrease in cardiac index (50%) during a 5-min test period of hepatic vascular occlusion can be used to identify the group of patients who require VVB. Other indications of the VVB include presence of pulmonary hypertension, impaired ventricular function from previous myocardial infarction, ischemic heart disease, and cardiomyopathy [38]. In patients with pulmonary hypertension, excessive fluid loading to compensate for hemodynamic changes during anhepatic phase may result in acute right ventricular dysfunction. Patients

with cardiomyopathy have impaired left ventricular function, and consequently a limited ability to generate adequate CO in the face of the increase in SVR during the anhepatic phase. These patients, too, may benefit from ameliorative effect of the preload associated with VVB. Some centers use VVB in patients with impaired renal function (i.e., hepato-renal syndrome) in order to prevent further kidneys damage during the anhepatic phase and to reduce the need for postoperative renal support. Among the advantages of VVB, some researchers listed the ability to reduce hemodynamic instability during anhepatic phase. It is useful in patients with pulmonary hypertension and cardiomyopathy who tolerate anhepatic period poorly. VVB has been shown to maintain intraoperative renal function [39, 40]. It also helps to maintain cerebral perfusion pressure in patients with acute fulminant failure by avoiding rapid swings in blood pressure, and, at least theoretically, may reduce blood loss [41]. However, VVB is not devoid of certain disadvantages. It does not guarantee normal perfusion of abdominal organs and lower limbs, since venous return never could be maintained at prebypass levels. The pump could only provide up to 2 L/min output (most commonly, only 1.5–1.8 L/min), which is, however comparable with low-to-normal levels of CO, cannot ensure the normal or even near-normal level of preload [42]. There is neither evidence of general(patient- and organ survival) outcome improvement, nor that it's use reduces or prevents the occurrence of postoperative renal failure [43]. VVB may exacerbate coagulation problems and cause excessive bleeding by inducing hemolysis, platelet depletion.

Graft reperfusion causes major hemodynamic changes along with possible substantial end-organ injury. These may include direct myocardial injury, resulting in tachy/bradyarrhytmias and cardiac arrest, profound vasoplegia, acute interstitial pulmonary oedema, leading to further RV overload/acute insufficiency, raise of pulmonary artery pressure (PAP) and CVP. Blood loss, hemodilution, hypovolemia, temperature drop, and rapidly developing lactic acidosis contribute to decreased sensitivity to catecholamines and efficiency of vasopressors. All these factors lead to rapid drop of SVR, resulting in a decrease of MABP with or without CO/CI decrease. Postreperfusion syndrome (PRS) was defined as a more than 30% decrease of MABP from that in the anhepatic stage, longer than for 1 min during the first 5 min after reperfusion of the liver graft [44–46].

In the postreperfusion period, the major factors of hemodynamic instability include ongoing blood loss, exacerbated by consumption coagulopathy in the face of very limited or almost nonexisting production of coagulation factors by the liver graft. Hypocalcemia, resulting from the effects of citrate-containing blood conservation solution, associated with transfusion of large amounts of RBC, exacerbates reduction of myocardial contractility caused by recent reperfusion. Acidemia, mostly due to lactic acidosis, substantially decreases efficacy of vaso-active agents.

## 4. Blood loss and coagulopathy management

### 4.1. Blood loss estimation and prediction factors

Blood loss during OLT is a well-known major factor of morbidity/mortality and overall hemodynamic instability, varying from just hundreds of ml up to dozens of liters. Predisposing

factors for major blood loss may include pre-existing + ongoing consumption and dilution coagulopathy (i.e., preoperative prothrombin time (PT), International normalized ratio (INR) and platelets numbers, factor V levels, etc.), MELD score >25, severe portal hypertension, "hostile abdomen" —postlaparotomy, re-do orthotopic liver transplant (OLT), long ischemia times, aged/marginal quality donor organ, donor-recipient organ size discrepancy, long, traumatic liver dissection, and surgeon-related factors.

Substantial number of studies reported no statistically significant correlations between blood loss and most of aforementioned parameters, particularly in respect to MELD score [47] and INR [48].

To date, blood loss and associated massive blood transfusion during OLTs remain difficult to predict [49]. Intraoperative blood salvage technique provides at least some way for blood loss estimation, with considerable approximation. Correspondent guidelines, based on calculations of hematocrit during blood loss (25–30%) and that of returned red blood cells by Cell-Saver (approximately 55–65% depending on Cell-Saver model), have been developed. Authors calculated estimated blood loss by multiplying the total volume of Cell-Saver returned RBCs by factor 3.4–4.0 [50, 51].

### 4.2. Coagulopathy: mechanisms and assessment

Of all the aforementioned factors, coagulopathy presents by far the most important and potentially most correctable problem, contributing to overall blood loss and, therefore, hemodynamic instability. Bleeding during OLT is multifactorial due both to surgical trauma and to coagulation defects. Coagulation defect in ESLD patients include impaired coagulation factor synthesis, dysfunction of coagulation factors, increased consumption, and fibrinolysis. Commonly, the levels of factor VII and protein C decrease first, followed by reductions in factors V, II, and X levels [52]. Platelet function is also affected by liver disease, and thrombocytopenia is common. Predisposing factors include hypersplenism secondary to portal hypertension, decreased thrombopoietin synthesis, immune complex-associated platelet clearance, and reticuloendothelial destruction [53].

During the dissection phase of the transplant, excessive bleeding is related to the technical difficulties during the liver dissection, and presence of portal hypertension, with large dilated collaterals [54].

During the anhepatic phase, coagulation factor synthesis is practically nonexistent, and ongoing factors consumption exacerbate the bleeding.

Right after graft reperfusion, profound coagulation abnormalities are very common. Factors that contribute to excessive bleeding in postreperfusion period include platelet entrapment in the sinusoids of the donor liver, a global reduction of all coagulation factors (mainly due to increased consumption, and partially due to hemodilution), and decreased level of antifibrinolytic factors [55, 56].

Method of thromboelastography (TEG) allows a rapid graphic assessment of the functional clotting status and degree of fibrinolysis. In various studies, the amount of RBCs and fresh

frozen plasma (FFP) usage has been significantly reduced when TEG monitoring that was compared to the conventional "clinician-directed" transfusion management [57, 58]. Although the usefulness of TEG in complex coagulation defects has been questioned [59], recent studies have shown, that the use of TEG can reduce the number of blood products transfused [58].

### 4.3. Hemotransfusion requirements and strategies

Blood transfusion therapy remains a critical component of anesthetic management and perioperative care in OLT. Multiple studies have shown a large variability in the use of blood products among different centers and among individual anesthesiologists within the same center [60]. The decision of when to transfuse RBCs, remains debatable. Some authors recommend keeping the hematocrit between 30 and 35%; others think it advisable and acceptable to maintain it between 26 and 28% [61, 62]. The modern trends have shown a substantial change from a transfusion of 10–20 units to 0–5.

The standard indication for fresh frozen plasma (FFP) infusion is coagulation defect treatment. FFP is expected to improve complex coagulation disorders in case of severe bleeding as it contains all coagulation factors and inhibitors. However, Freeman et al. [62] maintain that FFP administration is not essential during OLT, and that platelets and fibrinogen concentrates may be given when platelet count and fibrinogen level fall below 50,000 $mm^3$ and 1 g/L. In some centers, the trigger point is INR lower than two, which remains controversial. It has been shown that TE-guided coagulation defect management generally lowers the FFP amount. There is currently no consensus on the volume of FFP or rate of infusion required; in common practice, 10–15 mL/kg are usually administered. Because of the lack of universally accepted guidelines, the amount and timing of FFP administration during OLT are still guided by experienced clinical judgment, local practices, and coagulation tests (including TEG).

Although there is no consensus regarding the appropriate threshold value [64], platelet concentrates are frequently administered during OLT to address "oozing" on the operation field that likely could be attributed to the lack clot formation ability. Inter-center indications for platelet transfusion vary, but it seems that the current trend is to administer platelet transfusions pretty much regardless of the absolute PLT count.

It has been shown in many studies that the massive use of blood products during OLT is associated with increase in morbidity and mortality [65, 66]. It has been demonstrated that the intraoperative transfusion of red blood cells (RBCs) is associated with increase of postoperative mortality, specifically reduce survival rates at six months (63.8 vs. 83.3%) and at 5 years (34.5 vs. 49.2%), thus became a major prediction factor of mortality [59, 67, 68]. Higher intraoperative RBC transfusion requirements are associated with higher reintervention rates. Patients, who undergo reintervention, have three times higher mortality than those who do not have reinterventions [69, 70]. All blood products (RBCs, fresh frozen plasma (FFP), and platelets) have been shown to be negatively associated with graft survival at 1 and 5 years by univariate analysis [71]. Recent studies show that FFP and platelet transfusions are linked to the development of ALI/ARDS [71]. Pereboom et al. demonstrated, that platelet transfusion during OLTx is associated with increased postoperative mortality due to transfusion-related

acute lung injury (TRALI)/ARDS [63]. Intraoperative platelet transfusions have been identified as a strong independent risk factor for patient survival after OLT in addition to RBCs [72]. Studies have demonstrated that platelets are involved in the pathogenesis of reperfusion injury of the liver graft by inducing endothelial cell apoptosis. This effect is independent of ischemia-related endothelial cell injury [73].

### 4.4. Ways of blood loss reduction

Ways of blood loss reduction include surgical techniques such as Piggy-back technique with IVC preservation—partial Inferior vena cava (IVC) clamp, and anesthesia management options, such as maintaining the low CVP, minimal hemodilution with limited crystalloids infusion, and vasoactive agents use. Discussion of surgical techniques is beyond the scope of this review; however, anesthetic management options and techniques, intended to reduce blood loss during OLT are in the focus of discussion.

#### *4.4.1. Fluid management and "low CVP" paradigm*

Balanced fluid administration and maintaining relative hypovolemia have been advocated by many authors. A low CVP has been recommended to minimize blood loss during dissection stage of the liver transplantation. Massicotte et al. [74, 75] reported that maintaining a low CVP before the anhepatic phase was an efficient technique to decrease blood loss and transfusion rate. However, low CVP is associated with increased risk of complications, such as tissue hypoperfusion, development of lactic acidosis and renal failure, and also significant morbidity and mortality [76]. As it has been observed, increase in serum creatinine level, indications for dialysis, and 30-days mortality were higher in group of liver transplant patients, where CVP has been kept at low levels (around 3–5 sm$H_2O$), in order to avoid venous congestion of the graft. However, no supportive evidence has been found for decreasing CVP and effective circulating blood volume during OLT levels, currently accepted in some centers for liver resection [77]. Due to the lack of adequately powered, randomized, prospective controlled trials further investigations are needed to determine which patients would benefit from restrictive volume management in the intraoperative period of OLT.

#### *4.4.2. Blood salvage technique during OLT*

The use of intraoperative blood salvage and autologous blood transfusion has been for a long time an important method to reduce the need for allogeneic blood and the associated complications [78]. It has been demonstrated, that, for systematic use of Cell Saver salvaged blood in 75 OLT cases, retransfusion volume was enough and adequate in 65% of the cases [79].

The resultant hematocrit after Cell Saver processing ranges between 50 and 80% [80]. The safety of cell-salvaging procedure has been widely demonstrated [81]. Use of intraoperative autologous transfusion resulted in conservation of RBCs and reduction in exposure to homologous blood and blood components [82, 83]. Use of Cell Saver during OLT made it possible to recover up to 50% of blood loss [84]. Substantial reduction in FFP and a lesser reduction in platelet requirement have also been seen.

Nonetheless, blood-salvaging techniques during OLT are still being considered as controversial. Some studies have reported relatively higher blood loss, increased incidence of fibrinolysis, and cost rise [85, 86].The increased blood loss in recipients, receiving Cell Saver blood has been attributed to the release of fibrinolytic compounds from blood cells in the collected blood and/or from the transplanted liver [87]. These findings, however, have not dissuaded the anesthesiologists from using Cell Saver during OLTs; in fact, this method is gaining wider popularity, and becoming almost a standard of care in many centers around the world.

## 5. Vasoactive agents applied pharmacology and use in hemodynamic management during OLT

Hemodynamic instability during OLT due to blood loss, graft reperfusion, and postreperfusion vascular tone adjustment, substantial fluid shift oftentimes necessitates the use of vasoactive agents. Different vasopressors, such as dopamine, dobutamine, epinephrine, norepinephrine, phenylephrine, vasopressin, and, more recently, terlipressin and octreotide have been used for hemodynamic optimization and end-organ perfusion improvement during OLTs for decades [88, 89].

Norepinephrine and phenylephrine have a universal vasoconstrictor effect due to $\alpha$-receptor stimulation, thus effectively increasing systemic vascular resistance, while decreasing cardiac index, peripheral and portal blood flow [90–93]. However, norepinephrine in higher doses causes severe peripheral vasospasm and promotes metabolic (lactic) acidosis [88]. Phenylephrine increases SVR and MPAP, while it decreases CO/CI, peripheral, and portal BF [93], and does not affect portal VP during the dissection phase. CVP is often increased and does not seem to reflect cardiac filling [94].

Epinephrine and norepinephrine decrease liver and kidney tissue perfusion, thereby reducing lactate clearance, promote lactic acidosis, cause temporary alterations of hepatic macro- and microcirculation (return to baseline 2 h after onset of infusion). Dose-dependent progressive decline of hepatic macro- (33–75% reduction) and microcirculation (39–58% reduction) was found in transplanted livers. Norepinephrine has a direct constrictor effect on liver sinusoids, thereby reducing hepatic blood volume/flow and aggravating portal hypertension, and demonstrates effects similar to those of vasopressin effects on CO/CI and SVR [95], does not increase HBF, hepatic DO2 or VO2, and does not improve the hepatic lactate extraction ratio [96]. Vasopressin increases SVR, decreases MPAP; normalizes CO/CI, and potentially, CVP; maintains mean BP; decreases portal pressure, HBF, and systemic blood flow (SBF); improves impaired renal function; enhances diuresis, and thus improves Na balance and lactate elimination; enhances platelet aggregation; and increases levels of Profactor VIII and von Willebrand factor, and does not promote lactic acidosis. Its use after reperfusion, albeit having been shown beneficial by many authors, remains controversial, mainly due to splanchnic flow restriction effect with potential impairment of portal flow to the graft. Vasopressin has been demonstrated to have a dose-dependent vasoconstrictor effect on the peripheral vasculature with substantial SVR increases, while having little effect on heart rate, systemic arterial blood pressure, and CI in normotensive patients [97]. The ability of vasopressin to selectively

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constrict splanchnic vasculature, and thus decrease portal blood flow, is thought to constitute a physiological basis for variceal bleeding control by a higher vasopressin (0.4 U/min) dose [98, 99]. Vasopressin decreases portal vein pressure and flow in the native liver during liver transplantation [100]. Authors' own study has shown that use of low-dose vasopressin (0.04 U/min) infusion in an attempt to reduce blood loss seems to be a promising and a feasible technique. Vasopressin decreases portal vein pressure and blood flow in the native liver, as do terlipressin and octreotide [101]. A low-dose vasopressin (0.04 U/min) infusion apparently exerts only a minimal effect on the general hemodynamics. Low-dose vasopressin infusion is proved to be safe: to date, no cases of liver graft damage have been documented. To the contrary, cases where a high-dose of vasopressin (0.8 U) bolus, followed by a vasopressin infusion (4U/h) to attenuate refractory hypotension secondary to graft reperfusion, was used without causing any identifiable liver graft damage, have been reported [102]. Vasopressin has been shown to have a stimulation effect on lactate production by liver cells and adipose tissue in the septic model [103], and to be able to decrease blood loss during pre- and anhepatic phases of OLT (namely, EBL before graft reperfusion has been decreased by 50.2% [104] **Figure 1**)

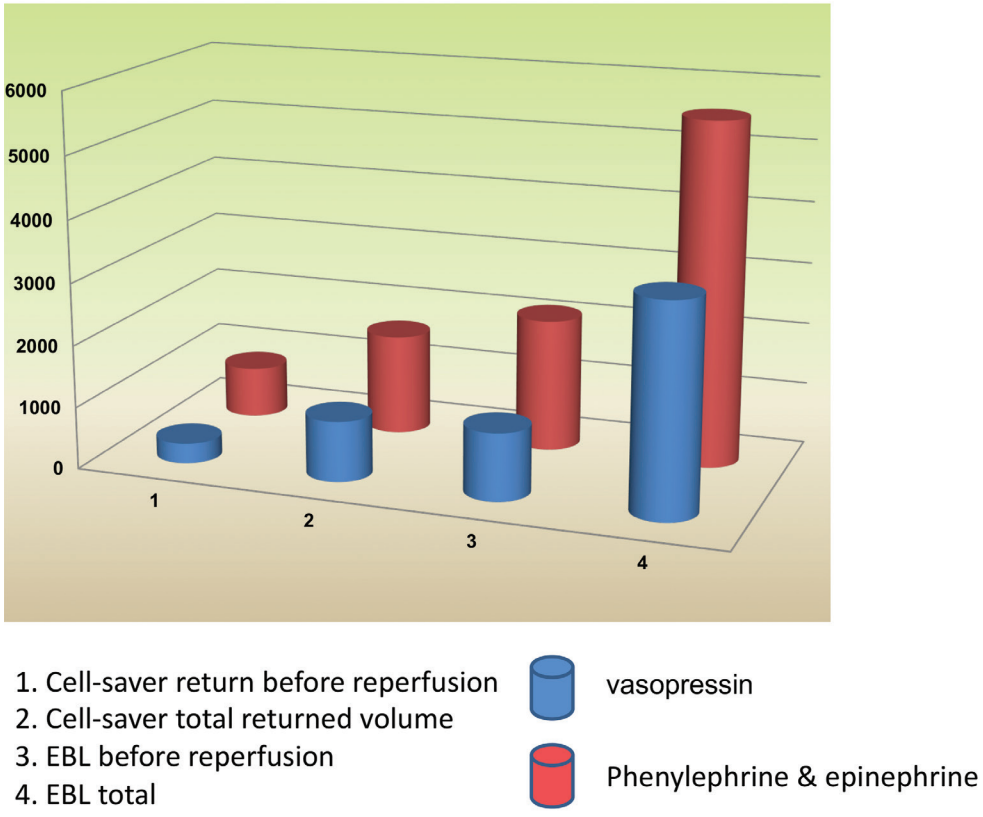


**Figure 1.** Blood loss decrease in pre-reperfusion stages of OLT: comparison of low-dose vasopressin and phenylephrine infusions.

### 5.1. Suggested algorithm of vasoactive agents used during anesthesia for OLT

Phenylephrine, epinephrine, norepinephrine, dopamine, and vasopressin are commonly used during different stages of OLT. The task of attaining hemodynamic stability sometimes dictates concomitant use of two or more vasoactive agents (**Figure 2**).

Intraoperative use of dopamine, 3 mcg/kg/min in OLT is intended to preserve and protect the adequate renal function, especially in cases of hepatorenal syndrome [105]. Higher rates of dopamine infusion, 5–10 to 20 mcg/kg/min, increase cardiac output and SVR. However, gaining CO/CI increase at the expense of tachycardia and, potentially, some rhythm disturbances makes dopamine a less desirable agent.

Early in the perunhepatic (dissection) stage of the surgery, phenylephrine infusion may be started, along with already running dopamine and low-dose vasopressin. Due to phenylephrine's almost purely $\alpha$-mimetic activity, its use actually addresses the low SVR problem, a main culprit for low MABP in majority of cases, provided that volume status correction and maintenance is being performed properly. In the majority of cases, phenylephrine infusion continues throughout the case. Providers in the other centers prefer and advocate early norepinephrine-only infusion be started, while others combine these agents [106].

Anhepatic stage often presents a challenge in terms of maintaining of hemodynamic stability. Rapid decrease in venous return; therefore, potential drop of CO, exacerbated by significant blood loss, usually necessitates more aggressive approach. Along with increase of norepinephrine (and phenylephrine, if its infusion is running along with the former), epinephrine may be added, with the purpose of using its $\beta$-stimulation activity. In preparation graft reperfusion,

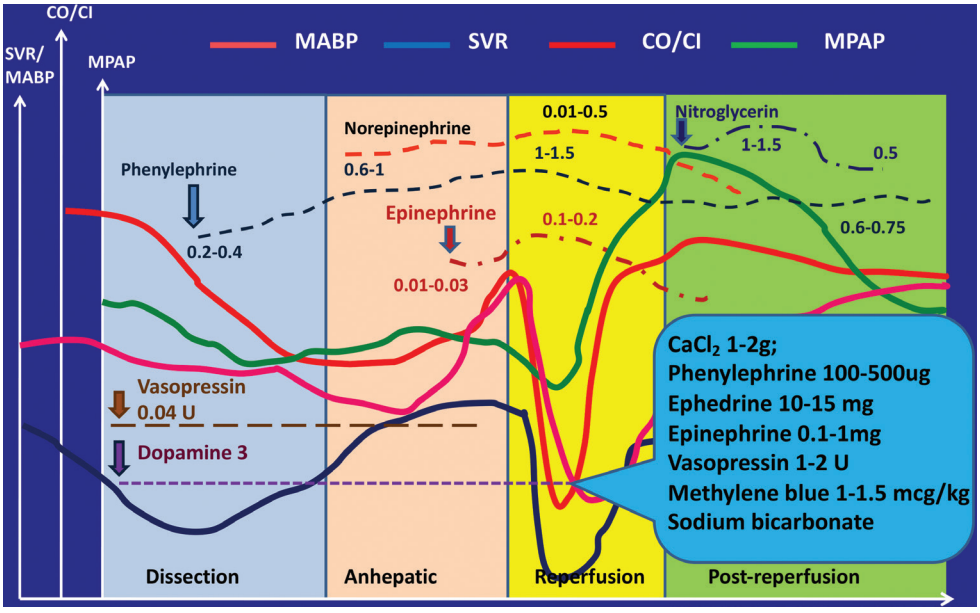


**Figure 2.** Use of different vasoactive agents throughout the whole of the OLT procedure.

some authors actually recommend "pretreatment" [107] with epinephrine and phenylephrine combination for postreperfusion syndrome prevention.

Graft reperfusion and postreperfusion syndrome presents a most significant challenge for hemodynamic management. Many different techniques and drug combinations has been tested and recommended for rapid hemodynamic recovery after liver graft reperfusion. Along with vasoactive agents and their combinations that are already in use by the time of a graft reperfusion, other agents has been successfully used (Figure 1). Vasopressin in small boluses, 1–2 U, may be highly efficient in opposing the significant and rapid decrease of SVR, and calcium chloride, up to 100 mg, may enhance inotropic effects of epinephrine [108]. Another agent, namely Methylene Blue, 2 mg/kg, has been reported as very efficient and "last resort" drug for prolonged and profound hypotension, refractory to treatment with other vasoactive drugs [109].

The presence of significant metabolic, mainly lactic, acidosis is a well-known cause of decreased vasoactive agent's efficiency [110]. To overcome hyporesponsiveness to vasopressors, sodium bicarbonate infusion may be necessary. THAM infusion provides a fast and efficient way of acidosis reversal and returning pH closer to the physiological range [111].

In certain cases, shortly after even seemingly uneventful graft reperfusion, PAP and CVP start to rise and graft congestion ensues. Reasons for this pulmonary pressure surge include postreperfusion left ventricle diastolic dysfunction as a result of direct myocardial injury, caused by free oxygen radicals containing metabolic substances, relative overload due to rapid transfusion of substantial amounts of blood products, interstitial pulmonary edema with PVR increase, and more. Graft congestion causes substantial perfusion and oxygen delivery impairment in the newly transplanted liver, that delays normal function restoration, specifically restart of coagulation components synthesis, which, in turn, exacerbates and prolongs the coagulation deficit. To address this problem, infusion rates of vasoactive drugs should be adjusted to the best possible balance of MAP and PAP, blood products transfusion rate (but not necessarily volume) should be decreased, diuretics (Furosemide) may be administered, and infusion of nitroglycerin, starting at 1 mcg/kg/min, may be commenced, as blood pressure tolerates. Nitroglycerin has proved to be an effective agent for treatment of pulmonary hypertension. It has been shown that nitroglycerin infusion resulted in pulmonary vascular resistance decrease by 43%, and mean pulmonary artery pressure decrease by 19% [112].

Hemodynamic management of postreperfusion stage of liver transplantation procedure consists of continuation of vasoactive agents infusion and usually involves a gradual decrease of infusion rates and also weaning from most aggressive vasopressors, like epinephrine. In substantial percentage of the cases, despite the adequate volume status restoration and coagulation defect complete reversal, the necessity for vasoactive drugs persists. Hemodynamic optimization continues well beyond the actual end of the surgery, oftentimes for a few days in critical care units.

Choice and dosage of vasoactive agents at every stage of OLT depend and should be guided by hemodynamic parameters. We suggest the allocation to all the patient population undergoing liver transplantation surgery, in three groups, according to hemodynamic parameters: compensated (MAP 80–100 mmHg, SVR > 600 dynes/s/cm$^5$), subcompensated (MAP 60–70 mmHg, SVR 300–600 dynes/s/cm$^5$), and decompensated (MAP <50 mmHg, SVR <200–250 dynes/s/cm$^5$)

Suggested algorithm of vasoactive agents use and dosage is summarized in **Table 1**.

| OLT stage | Hemodynamics | | | | | |
|---|---|---|---|---|---|---|
| | MAP 80–100, SVR>600 | | MAP 60–70, SVR 300–600 | | MAP<50, SVR <200–250 | |
| | Agent | Dose | Agent | Dose | Agent | Dose |
| Dissection | Dop | 3 | Dop | 3 | Dop | 5–10 |
| | Phen | 0.2–0.4 | Phen | 0.4–0.6 | Phen | 0.6–1.0 |
| | Vas | 0.04 | Vas | 0.04 | Vas | 0.04–0.08 |
| | | | | | NE | 0.01–0.03 |
| An-hepatic | Dop | 3 | Dop | 3 | Dop | 5–10 |
| | Phen | 0.2–0.4 | Phen | 0.4–0.8 | Phen | 0.8–1.2 |
| | Vas | 0.04 | NE | 0.01–0.03 | NE | 0.04–0.08 |
| | | | Vas | 0.04 | Epi | 0.01–0.03 |
| | | | | | Vas | 0.04–0.08 |
| Reperfusion | Dop | 3–5 | Dop | 3–5 | Dop | 3–5 |
| | Phen | 0.2–0.6 | Phen | 0.6–0.8 | Phen | 0.8–1.2 |
| | Ca | 500 | NE | 0.04–0.08 | NE | 0.06–0.1 |
| | | | Epi | 0.02–0.04 | Epi | 0.04–0.08 |
| | | | Ca | 1000 | Vas | 3–5 |
| | | | Vas | 1–2 | Ca | 1000–2000 |
| | | | | | MB | 1–1.5 |
| | | | | | Bic | 50–100 |
| Post-reperfusion | Dop | 3 | Dop | 3 | Dop | 3–5 |
| | Phen | 0.02–0.06 | Phen | 0.4–0.8 | Phen | 6–1.0 |
| | | | NE | 0.02–0.04 | NE | 0.08–0.1 |
| | | | | | Epi | 0.02–0.04 |

Dop—dopamine; Phen—phenylephrine; NE—norepinephrine; Epi—epinehrine, all dosage in mcg/kg/min; Vas—vasopressin, units/min; Ca—calcium chloride, mg; MB—Methylene Blue, mg/kg; Bic—sodium bicarbonate, mEq.

**Table 1.** Algorithm of vasoactive agents use and dosage during OLT.

## 6. Conclusion

Hemodynamic optimization during liver transplant surgery presents very complex, challenging, sometimes formidable task, many aspects of which remain unclear, thus warrant further research. A wide variety of anesthetic techniques and standards, institutional policies,

hemodynamic triggers for vasoactive agents use and transfusion thresholds, arriving at the even nation-wide consensus, let alone worldwide, remain extremely difficult, if not mere a unrealistic task. Nonetheless, introduction of comprehensive guidelines, based on most common clinical practices and realities of perioperative hemodynamic management appears to be not only conceivable but rather timely and a necessary enterprise. Once introduced, such guidelines may lay the ground for successful and safe intra and perioperative practices and also provide support for much-needed research efforts in this complicated area of transplant anesthesia practice.

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Chapter 10

# Management of Hepatocellular Carcinoma in the Setting of Liver Cirrhosis

Alexander Giakoustidis and Dimitrios E. Giakoustidis

Additional information is available at the end of the chapter

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**Abstract**

Cirrhosis is an increasing cause of morbidity and mortality in more developed countries, being the 14th most common cause of death worldwide. Hepatocellular carcinoma (HCC) consists a significant health issue worldwide, responsible for more than 1 million deaths annually. The incidence and mortality rates vary across different geographical areas. Between 60 and 90% of HCC patients already have liver cirrhosis, attributed mainly to chronic hepatitis B and C, alcohol abuse, and non-alcoholic fatty liver disease (NASH). The surgical management of HCC in the setting of liver cirrhosis with curative intent includes liver resection, ablation or microwave coagulation, and liver transplantation (LT). Liver resection in a cirrhotic liver with HCC is associated with lower survival rates compared with liver transplantation (LT), depending on the diseases' stage but on the contrary liver resection could be potentially offered in a larger population compared to liver transplantation. One of the biggest limitations of liver resection is the risk of tumor recurrence, which is high, and it may exceed 70% 5 years after the procedure. Liver transplantation is considered the best treatment for hepatocellular carcinoma at early stages because it removes the tumor as well as the underlying cirrhotic liver.

**Keywords:** liver resection, liver transplantation, HCC, cirrhosis, RFA, TACE

## 1. Introduction

Cirrhosis is an increasing cause of morbidity and mortality in more developed countries, being the 14th most common cause of death worldwide. The natural history of cirrhosis is initially compensated and is asymptomatic progressing into decompensated cirrhosis

with portal hypertension and liver dysfunction and in the development of hepatocellular carcinoma (HCC).

Hepatocellular carcinoma consists a significant health issue worldwide, responsible for more than 1 million deaths annually. The incidence and mortality rates vary across different geographical areas [1, 2]. Between 60 and 90% of HCC patients already have liver cirrhosis, attributed mainly to chronic hepatitis B and C, alcohol abuse, and non-alcoholic fatty liver disease (NASH). In the past, HCC was usually diagnosed late during the course of the liver disease, and consequently, the vast majority of patients had a poor prognosis at diagnosis. Survival is poor, and high recurrence rates after treatment were exhibited regardless of treatment. Currently, the implementation of screening programs especially for chronic virus hepatitis, and advances in radiological assessment, leads to an increasing proportion of patients being diagnosed within early stage of HCC. The surgical management of HCC in the setting of liver cirrhosis with curative intent includes liver resection, ablation or microwave coagulation, and liver transplantation (LT).

## 2. Hepatocellular carcinoma staging

Cancer staging should serve to select the appropriate primary and adjuvant therapy, to estimate the prognosis, and also to assist in the evaluation of the results of treatment and this is also applicable in HCC [3, 4]. The EASL panel of experts recommended the consideration of four-related aspects: tumor stage, degree of liver function impairment, general condition of the patient, and treatment efficacy [5]. In the past, the Okuda classification [6] has been widely applied in HCC patients, and it included parameters related to the liver functional status like albumin, ascites, and bilirubin. The Cancer of the Liver Italian Program (CLIP) score [7] was proposed and validated [8]. It combines four variables that provide a seven-stage classification system and was more discriminatory compared with Okuda stage and TNM. Groups from Asia published different survival rates compromising its external validation [9]. The Barcelona-Clinic Liver Cancer (BCLC) staging system [10] was proposed by the Barcelona group on the basis of the results obtained from cohort and RCT studies. It consists a staging classification that uses variables related to performance status, tumor stage, liver functional status, characteristic of the tumor, vascular invasion, and the presence of portal hypertension (PH). This BCLC classification system has become a widely accepted algorithm for all HCC patients in earlier disease, linking their current status prognosis with treatment recommendations. Recently, a new staging system was proposed from the Hong Kong group [11]. The Hong Kong Liver Cancer (HKLC) used four prognostic factors in the treatment of HCC, the Eastern Cooperative Oncology Group performance status (ECOG PS), Child-Pugh grade, liver tumor status, and presence of extrahepatic vascular invasion/metastasis. Liver tumor status was a composite factor of the size of the largest tumor in the liver, number of tumor nodules, and the presence or absence of intrahepatic vascular invasion. The authors support that the HKLC staging classification has the potential to provide better prognostic classification than BCLC staging and may be more effective in identifying patients suitable for more aggressive treatments, hence yielding a better survival outcome.

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## 3. Liver resection vs. TACE and RFA

Liver resection when it is feasible, in a cirrhotic liver with HCC, is associated with lower survival rates compared with liver transplantation (LT), varying from 35 to 62% at 3 years and from 17 to 50% at 5 years, depending on the diseases' stage but on the contrary liver resection could be potentially offered in a larger population compared to liver transplantation. One of the biggest limitations of liver resection is the risk of tumor recurrence, which is high, and it may exceed 70% 5 years after the procedure. Hepatic resection tends to be applicable only in patients with cirrhosis that is classified as Child-Pugh class A or B and with mild portal hypertension. The application of palliative therapies like radiofrequency ablation (RFA), microwave coagulation (MC) and transarterial chemoembolization (TACE) is frequently limited by impaired hepatocellular function, severe portal hypertension, or multiple tumor nodules.

Huang et al. [12] in a large randomized trial of 230 patients within the Milan criteria (BCLC stage A) compared surgical resection and radiofrequency ablation for HCC patients indicating a favorable outcome for surgically treated patients. Wang et al. in their meta-analysis evaluated three randomized and 25 nonrandomized trials, and they confirmed the long-term superiority of surgical treatment [13]. In another meta-analysis by Kapitanov et al. and taking into account the limited available literature and prospective studies, they concluded that liver resection shows significantly improved long-term survival compared to TACE in cirrhotic patients with BCLC stage A and B HCC. T. Utsunomiya et al. conducted a large prospective multicenter trial and demonstrated clear superiority for hepatic resection when compared to TACE and RFA for patients with Child-Pugh stage A and B liver cirrhosis and stage II HCC (JIS scores 1 and 2) [14]. Peng et al. [15] showed that even for patients with portal venous tumor, thrombus liver resection improves long-term survival compared to TACE as long as tumor thrombosis was confined to the liver. This effect vanished in the presence of extensive tumor thrombosis into the portal venous confluence and the superior mesenteric vein. Zhong et al. [16] demonstrate clear superiority for hepatic resection versus TACE in terms of patient survival. They analyzed an impressive total number of 1259 of patients with the vast majority of cases being hepatitis-B positive. Limitations of the study were a rather heterogeneous patient collective and a mean patient age and tumor size being both greater in the TACE group. For this reason, matched-pair analysis was performed between TACE and resection patients with identical demographics confirming the positive overall results for surgically treated patients.

Laparoscopic liver resection (LLR) consists a contemporary surgical approach in the management of hepatocellular carcinoma with or without liver cirrhosis. The indications for LLR have changed substantially since its introduction. In the beginning, it was limited to benign diseases, while gaining increased knowledge and experience of the procedure, its indications have expanded to malignant diseases, including HCC and colorectal liver metastasis [17]. However, laparoscopy has been limitedly used for liver resection due to the risk of air embolism and the difficulty of parenchymal dissection and bleeding control [18]. Therefore, LLR has been frequently performed for tumors superficially located in the anterolateral segments

[19]. Liver cirrhosis consists a substantial risk factor for developing postoperative complications following hepatectomy. Severe blood loss or prolonged ascites after major hepatectomy, especially by open surgery, can occur by interruption of collateral circulation in the parietal wall and surrounding ligamentsin patients with liver cirrhosis [20] and may prolong the postoperative hospital stay or induce hepatic failure in some patients. However, LLR may minimize the reduction in collateral and lymphatic flow caused by laparotomy and mobilization [21, 22]. The benefits of LLR in liver cirrhosis include enhanced recovery, less postoperative pain, and potentially less postoperative complications. Other important advantages of LLR in patients with liver cirrhosis are the lower incidences of postoperative liver failure and ascites due to minimal invasiveness of LLR, which helps to preserve collateral circulation. Therefore, laparoscopic hepatectomy may be a good option in patients with cirrhosis [23].

## 4. Down-staging and bridge therapies

### 4.1. TACE

Down-staging in HCC patients includes but not limited to TACE, radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), microwave coagulation (MC), resection, and radiation [24]. The objective of down-staging is to decrease the tumor size and/or number of nodules in those patients that initially are presenting with tumors beyond the acceptable criteria for liver transplantation in different centers. The response to different DS treatment has to be based on radiological measurement of tumor characteristics. The EASL HCC guidelines suggested, and this was also endorsed by the AASLD guideline, that assessment of tumor response should consider only the area of viable tumor [25], defined by arterial enhancement on a radiological contrast study modified response evaluation criteria in solid tumors (mRECIST).

Prospective studies showed that survival after liver transplantation in patients with large tumor burden successfully treated by down-staging was similar to survival in patients who initially met the criteria for transplantation [26]. There is currently no well-defined upper limit for size and number of lesions as eligibility criteria for down-staging, although the presence of vascular invasion and extrahepatic disease is generally considered absolute contraindications.

The role of DS has been ambiguous concerning the overall and recurrence-free survival post-transplantation. In the case that complete tumor necrosis with locoregional therapy is achieved, this is associated with better survival. A multicenter case-control study compared matched patients with TACE (100) and without TACE (100) [27] showed that survival rates 5 years after OLT were similar 59.3% versus 59.4%, respectively. In addition, there were fewer recurrences in the TACE group although this was not statistically significant. Moreover, the waiting times were short, and the median number of TACE procedures was only 1, and this may impact negatively the detection of any advantage for TACE.

Comparisons of the dropout rates of treated and untreated patients are limited with the existing data. Yao et al. from the UCSF analyzed 70 patients a proportion of them having pretransplant therapy either TACE or ablation, and this was associated with a significantly lower risk

of dropout. Disadvantage of the study was that the population was heterogeneous regarding the disease stage, and the criteria for treatment were influenced by external factors [28]. Another study from Toronto including 74 patients identified a difference in tumor-related dropout that became apparent only after 300 days [29].

Drug-eluting beads loaded with chemotherapy agents are delivered into the tumor through the feeding artery. Chemotherapy agents are released gradually, so systemic side effects are reduced, and tumor drug delivery is enhanced. The PRECISION study compared conventional TACE with DEB for the treatment of 212 patients with Child-Pugh A or B cirrhosis and unresectable HCC [30]. Subpopulation analysis revealed that patients with Child-Pugh B cirrhosis or bilobar tumor disease showed a better response to DEB. In addition, the overall DEB was better tolerated than conventional TACE. While it appears that DEB might be better tolerated than conventional TACE, more extensive data are needed.

### 4.2. RFA

The use of RFA as a bridge to transplantation in HCC patients is also applicable. It has been reported complete tumor necrosis at pathological evaluation of the explanted liver in 47–75% of cases, with a mean value of 58% [31–35]. Different rates of complete necrosis ranges have been observed between 50 and 78% in HCCs up to 3 cm and between 13 and 43% in larger neoplasms, respectively [31–33]. Furthermore, in two studies, a tumor size larger than 3 cm was the only risk factor found for HCC recurrence after treatment [31–33]. Analysis of the largest available series of HCC patients awaiting LT regarding RFA-related complications showed the safety of the procedure. From five large series, we could see that the mean rate of post-ablation major complications was below 5% [31–36], and in addition, the risk of tumor seeding at the level of the abdomen wall appears to be low.

### 4.3. Liver resection

Belghiti [37] proposed that resection can be used as an alternative treatment option for HCC or before LT as "down-staging" procedure. Liver resection can be used as a primary therapy in patients with HCC and well-preserved liver function, with LT reserved as a "salvage" therapy for patients who developed recurrence or liver failure. Moreover, resection can be used as an initial therapy in order to select patients whose explants pathology would be favorable for LT. Resection could also be used as a "bridge" therapy for patients who have been already enlisted for LT. Whether resection or LT should be the treatment of choice for small HCC in patients with preserved liver function is a hot issue and still in debate. Long-term overall survival after resection or transplantation appears comparable in a well-selected population with HCC within the Milano criteria [37–39]. LT has the advantage of increased disease-free survival compared with liver resection, but its use is limited by shortage of liver organs. It has been proposed by the group of Belgiti but also from other groups that resection as the first-line treatment for patients with small HCC with preserved liver function, followed by salvage transplantation only for recurrence or liver failure, would feasible in a large proportion of HCC patients [37–39].

Considering emergency LT after resection as center, policy would require a strict selection of the candidate with clear and strong indicators of irreversible postoperative liver insufficiency. Patients with liver failure due to massive necrosis of the remnant liver or those with uncontrollable bleeding are easy to be identified, but it is unclear and very difficult to ascertain the irreversibility of liver insufficiency in all settings. A significant increase in international normalized ratio (INR) and serum bilirubin within the first postoperative days is a common characteristic of extended resection making identification and selection of patients in need for early liver transplantation tricky. It is documented that, in the absence of any treatable complication, the lack of significant improvement on postoperative day 5 may lead to strongly considering rescue transplantation [40].

Poon et al. [38] proposed liver resection for HCC lesions in selected patients eligible for LT and to reserve LT for those who develop recurrence or deterioration of liver function. This approach, which proposes resection as a bridge treatment to prevent tumor progression during the waiting period, looks attractive but has not been studied well, especially with prospective studies and needs external validation of published data from the various transplant centers. As major concern from transplant surgeons is that prior liver resection especially if done in no-specialized centers could complicate the operative transplant procedure, increase the risk of postoperative complications, and finally compromise results and impair the survival advantage of transplantation over resection alone.

## 5. Liver transplantation

Liver transplantation is considered the best treatment for hepatocellular carcinoma at early stages because it removes the tumor as well as the underlying cirrhotic liver. However, as a result of organ shortage, it is anticipated that transplantation to HCC patients will be performed with an expected five-year post-transplantation survival of greater than 50%, and, in most programs, an expected five-year post-transplantation survival similar to survival achieved after liver transplantation for benign liver diseases (i.e., 70%).

In 1996, Mazzaferro et al. [41] conducted a prospective cohort study defining restrictive selection criteria (Milan Criteria (MC)) that led to improved survival for transplant patients compared with any other previous experience with transplantation for HCC. Adopting the MC demonstrated a five-year survival of 70% after LT [41]. The survival outcome of MC is comparable to LT in benign diseases and given that this excellent outcome MC has been established from most liver societies (EASL and AASLD guidelines) as the golden standard in selecting HCC patients for liver transplant [42, 43].

In 2001, Yao et al. from University of California San Francisco (UCSF) [44] demonstrated a tumor recurrence rate of little more than 10% and survival rates exceeding 70% in T1, T2 and T3 tumors. The new criteria included solitary tumors smaller or equal to 6.5 cm in size or three or fewer tumors with the largest diameter not exceeding 4.5 cm and the total tumor diameter being less or equal to 8 cm and became known as the UCSF criteria.

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Alternative criteria have been proposed by other centers. These include criteria from the Asan Medical Center in Korea [45], from Hangzhou, China [46], the University Clinic of Navarra in Spain [47], Kyoto, Japan [48]. All use different criteria in terms of number of nodules and size and in addition try to implement some biological criteria like $\alpha$-FP, protein induced by vitamin K absence II (PIVKA II) and other. Unfortunately, none of these criteria have been externally validated in order to get wider acceptance.

In 2009, the Metroticket was introduced by Mazzafero et al. [49]. The Metroticket introduced the logic that the further you expand HCC staging criteria for LT, this would impact negatively the outcome in terms of higher recurrence rates and poorer overall survival. This model potential could be a simple predictive model for estimating the survival of patients undergoing LT with tumors exciding the Milan criteria in number and size of the tumors.

High $\alpha$-fetoprotein (AFP) levels are predictive of poor prognosis in non-transplant patients, and AFP levels greater than 1000 ng/mL have been associated with a high risk of recurrence in the University of California, San Francisco (UCSF), experience [44] after liver transplantation.

AFP value is proposed as a good indicator in selecting HCC patients for LT [50, 51]. In the non-transplant patients, an elevated AFP is a marker of advanced disease. It has been proposed that an increase in AFP levels might be an indicator of tumor aggressiveness including differentiation degree and vascular invasion and, consequently, lead to a higher risk of tumor recurrence. Toso et al. [52] analyzed adult recipients in the Scientific Registry of Transplant Recipients. In the multivariate analysis, it was shown that high AFP levels and TTV >115 $cm^3$ were associated with poor long-term survival.

Duvoux et al. [53] in a French multicenter study showed that AFP levels strongly correlated with the pathologic features of HCC. Based on the analysis of 453 explanted livers, they found that increased AFP levels were associated with vascular invasion and loss of differentiation.

Living Donor Liver Transplantation (LDLT) consists of an alternative option to Deceased Donor Liver Transplantation (DDLT). Special consideration regarding LDLT for HCC is required, since patients for LDLT are not dependent of the cadaveric donor pool, but bring their "own" liver graft. It is important to stress that the application of strict eligibility criteria similar the one required with cadaveric grafts for patients with HCC might not be necessary. However, survival benefit to the recipient should be substantial, and the risk to the donor must be incorporated into the centers policy, since it is clearly unethical to expose a donor to a significant risk of morbidity or mortality. Generally, similar criteria apply to patients undergoing DDLT or LDLT. For patients subjected to either DDLT or LDLT for HCC within MC, similar outcomes have been documented [54, 55]. Asian groups have proposed different policies concerning different criteria for LDLT in the setting of HCC. The Tokyo group applies the 5–5 rule (number of tumors not exceeding 5 and maximum tumor diameter not exceeding 5 cm); the Kyoto group the 10–5 rule (number of tumors not exceeding 10; each tumor not exceeding 5 cm) in combination with the biological tumor marker PIVKA (or DCP) (not exceeding 400 mAu/ml), and finally, the Seoul group adopts an intermediate policy with limiting the number of tumors not exceeding 6 and the maximum tumor diameter not exceeding 5 cm. All three groups obtained around 85% 3–5 years disease free survival (DFS) survival rates.

In the West, LDLT is often stretched in patients who do not strictly meet the Milan criteria for MELD exception points and have tumors with a probable worse prognosis. Updated re-analyzed data of the A2ALL cohorts concluded that "differences in tumor characteristics and management of HCC in patients who received LDLT likely accounted for the higher HCC recurrence rates observed in their LDLT group."

Systematic review analysis by Grant et al. [56] suggests that DFS is worse after LDLT compared with DDLT for HCC. Decreased DFS may eventually translate to decreased OS, and it is advisable that the increased risk of recurrence should be communicated to all potential donors and recipients who are considering LDLT for HCC.

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Chapter 11

# Impact of Glyoxalase-I (Glo-I) and Advanced Glycation End Products (AGEs) in Chronic Liver Disease

Marcus Hollenbach

Additional information is available at the end of the chapter

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**Abstract**

Inflammation caused by oxidative stress (ROS) is a main driver for development of chronic inflammatory liver disease leading to fibrosis and cirrhosis. An important source of ROS constitutes methylglyoxal (MGO). MGO is formed as a by-product in glycolysis, threonine catabolism, and ketone bodies pathway leading to formation of advanced glycation end products (AGEs). AGEs bind to their receptor for AGEs (RAGE) and activate intracellular transcription factors, such as nuclear factor-κB (NF-κB), resulting in production of pro-inflammatory cytokines and ROS. The enzymes glyoxalase-I (Glo-I) and glyoxalase-II (Glo-II) form the glyoxalase system and are essential for the detoxification of methylglyoxal (MGO). This chapter highlights Glo-I and (R)AGE in chronic liver disease with focus on fibrosis and cirrhosis. AGEs and RAGE have been shown to be upregulated in fibrosis, and silencing of RAGE reduced the latter. In contrast, recent study highlighted reduced expression of Glo-I in cirrhosis with consecutive elevation of MGO and oxidative stress. Interestingly, modulation of Glo-I activity by ethyl pyruvate resulted in reduced activation of hepatic stellate cells and reduced fibrosis in $CCl_4$ model of cirrhosis. In conclusion, Glo-I and R(AGE) are important components in development and progression of chronic liver disease and constitute interesting therapeutic target.

**Keywords:** ethyl pyruvate cirrhosis, fibrosis, methylglyoxal, AGEs

## 1. Introduction

Oxidate stress (reactive oxygen species, ROS) with consecutive and repetitive inflammation is responsible for development of chronic liver disease. Different etiologies of liver disease lead to damage of hepatocytes, release of pro-inflammatory cytokines, and finally activation

of hepatic stellate cells (HSC). Activated HSC transform to myofibroblasts and lead to deposition of collagen, which in turn result in fibrosis and finally cirrhosis. Several molecular mechanisms are involved in this complex interplay, nevertheless the critical step is the activation of HSC by ROS. This chapter focuses on the glyoxalase-I (Glo-I) and related advanced glycation end products (AGEs) with their receptor for AGEs (RAGE) playing an important role in generation and detoxification of ROS. Current knowledge of Glo-I and (R)AGE in chronic liver disease with key aspect to fibrosis and cirrhosis will be highlighted.

## 2. Pathogenesis of fibrosis and cirrhosis

End-stage liver diseases are mainly caused by viral hepatitis, alcoholism, nonalcoholic fatty liver disease or steatohepatitis (NAFLD/NASH), or rare autoimmune and hereditary disorders. The followed repetitive liver injury caused inflammation, finally resulting in fibrosis and irreversible cirrhosis. Thereby, liver cirrhosis belongs to the global burden of disease responsible for more than one million deaths p.a. [1]. In cirrhosis, altered liver anatomy and reduced liver function are pathognomonic. Development of cirrhosis is characterized by the appearance of regenerative nodules, hepatocyte ballooning, accumulation of fibrotic tissue, disturbed microcirculation, angiogenesis and sinusoidal collapse with defenestration and development of a basement membrane [2]. These alterations of liver architecture lead to reduced liver function and elevation of intrahepatic resistance demonstrated by increased portal pressure with development of ascites and esophageal varices [3, 4]. Nevertheless, portal hypertension is being caused by both structural alterations of liver microarchitecture and hepatic endothelial dysfunction. The latter is characterized by an imbalance of vasoactive components. In fact, there is an hyperresponsiveness and overproduction of vasoconstrictors (mainly endothelin-1 (ET-1)) and an hyporesponsiveness and reduction of vasodilators (mainly nitric oxide (NO)) in the vascular bed of the liver [5–7]. Despite this hypoactive endothelium in hepatic microcirculation, portal hypertension leads to arterial vasodilation, formation of collateral vessels, and hyporesponsiveness to vasoconstrictors due to hyperactive endothelium in splanchnic and systemic circulation with increased NO production. Finally, these alterations result in elevated blood flow to portal vein and a vicious circle of disease [8–11].

The underlying molecular mechanism for development of fibrosis, cirrhosis, and portal hypertension has been intensively investigated over the last decades. Since the liver is formed by parenchymal cells (mainly hepatocytes (HEP)) and nonparenchymal cells (Kupffer cells (KC), hepatic stellate cells (HSC), and liver sinusoidal endothelial cells (LSEC)), both are involved in the development of fibrosis and cirrhosis. Nevertheless, HSC are the main cell type responsible for accumulation of fibrosis and increased intrahepatic vascular resistance. HSC are pericytes surrounding the sinusoids in the space of Disse. HSC are quiescent but became activated upon various stimuli and transform to myofibroblasts [12]. This activation process is a complex interplay between parenchymal and nonparenchymal cells and triggered via inflammatory processes [13]. For instance, deleterious agents (alcohol, LPS) have direct hepatotoxic effects to hepatocytes and trigger the production of reactive oxygen species (ROS). The release of ROS, DNA, and damage-associated molecular pattern (DAMP) leading

to activation of KC and innate immune system followed by subsequent production of pro-inflammatory cytokines such as TNF-α and IL-6 as well as pro-fibrotic factors [14–16]. Also, alcohol consumption increases permeability of the gut resulting in increased levels of portal endotoxins (LPS) with consecutive activation of KC resulting in liver injury and inflammation [17, 18]. Furthermore, inflammation triggers the classical complement pathway activation via C1q [19], followed by production of pro-inflammatory cytokines, and inhibits components of innate immune system. As a consequence of these induced inflammatory processes, activated KC stimulate HSC subsequently leading to fibrosis [20]. This stimulation can result directly by the deleterious agent [21] or via transforming growth factor beta (TGF-β)-dependent mechanisms [22] leading to secretion of TNF-α, IL-6, TIMP-1, MCP-1, collagen-I, and α-SMA [23–25] and finally collagen deposition.

As mentioned above, pro-inflammatory factors (TNF-α, IL-1β, IL-6) are also involved in the activation of HSC. In this regard, activation of the transcription factor nuclear factor-κB (NF-κB) and subsequent overexpression of pro-inflammatory cytokines are important pathways. NF-κB, thereby, is activated by growth factors, cytokines, bacterial and viral factors, and ROS and regulates by itself pro-inflammatory cytokines (like COX-2 or IL-6) [26, 27].

Beside the production of collagen and accumulation of fibrotic tissue, HSC are involved in increased intrahepatic vascular resistance not only via structural changes. Transformation of HSC to myofibroblasts was accompanied by stimulation of rho kinase leading to activation of contractile filaments of HSC and subsequently vasoconstriction of sinusoids [28].

Another key player in the development of fibrosis comprises LSEC. They form the first line of defense protecting the liver from injury. Inflammation by LPS or ROS resulted in dysfunction of LSEC [29] indicated by disturbed sinusoidal microcirculation, defenestration, hypoxia, and pathological angiogenesis [30]. In contrast, both direct deterioration of LSEC and vasoconstriction of HSC result in impaired release of vasodilators from LSEC leading to a vicious circle of disease. In this regard, disturbed regulation of NO production in cirrhosis depends on activity of endothelial NO synthase (eNOS) and increased degradation due to phosphodiesterases, that is, PDE-5 [31]. Although eNOS expression is upregulated in sinusoidal area in cirrhosis, eNOS activity has been shown to be reduced by caveolin-eNOS binding [32] and was diminished by several post-translational modifications of the endothelial NO synthase (eNOS) [9]. In contrast, in splanchnic circulation, eNOS is upregulated [9] with increased enzyme activity in portal hypertension and regulated by phosphorylation of protein kinase B (Akt) [33]. Beside upregulation of eNOS, production of NO is also related to induction of the inducible form of the NO synthase, iNOS. iNOS is mainly stimulated by the presence of endotoxin and pro-inflammatory cytokines, all of whom occur in development of cirrhosis [34]. Indeed, recent study showed stimulation of iNOS rather than eNOS in splanchnic circulation by LPS, indicating an important role of iNOS in portal hypertension after bacterial translocation to mesenteric vessels [35]. Finally, all these alterations result in a hyperdynamic circulation with elevated blood flow to portal vein and further increase of portal pressure [8–10].

In conclusion, cirrhosis demonstrates the end stage of liver disease with disturbed liver architecture and impaired liver function. Generation of ROS and stimulation of various inflammatory pathways are critical steps in activation of HSC as the main driver for fibrosis. Despite

these findings, the use of antioxidants (vitamin E, N-acetylcysteine, coenzyme Q, and others) in patients with alcoholic liver disease has failed to show an efficacy in improving disease conditions [36–38].

## 3. Glyoxalase system and R(AGE)

An important role in regulation and formation of ROS and oxidative stress comprises the glyoxalase system. This enzymatic system was first discovered in 1913 [39] and constitutes two cytosolic enzymes, glyoxalase-I (Glo-I, EC 4.4.1.5) and glyoxalase-II (Glo-II, EC 3.1.2.6.). Glo-I is responsible for the catalytic conversion of $\alpha$-oxo aldehydes, for instance, methylglyoxal (MGO), into the hemithioacetal S-D-Lactoylglutathione using L-glutathione (GSH) as a cofactor. Further substrates of Glo-I are hydroxypyruvaldehyde, hydroxypyruvate aldehyde phosphate, glyoxal, phenylglyoxal, 4,5-dioxovalerate, alkyl and arylglyoxales [40–43]. Glo-II hydrolyzes the reaction of S-D-Lactoylglutathione to $H_2O$ and D-lactate with regeneration of GSH (**Figure 1**). Thereby, Glo-I demonstrates the rate limiting step [42, 44], and Glo-II is of subordinate interest in inflammatory research.

MGO is the main substrate of Glo-I [45] and has been described as a reactive carbonyl compound that is formed as a by-product in glycolysis [46], ketone body metabolism, and threonine catabolism [47–49]. MGO leads to cell cytotoxicity in high concentrations through

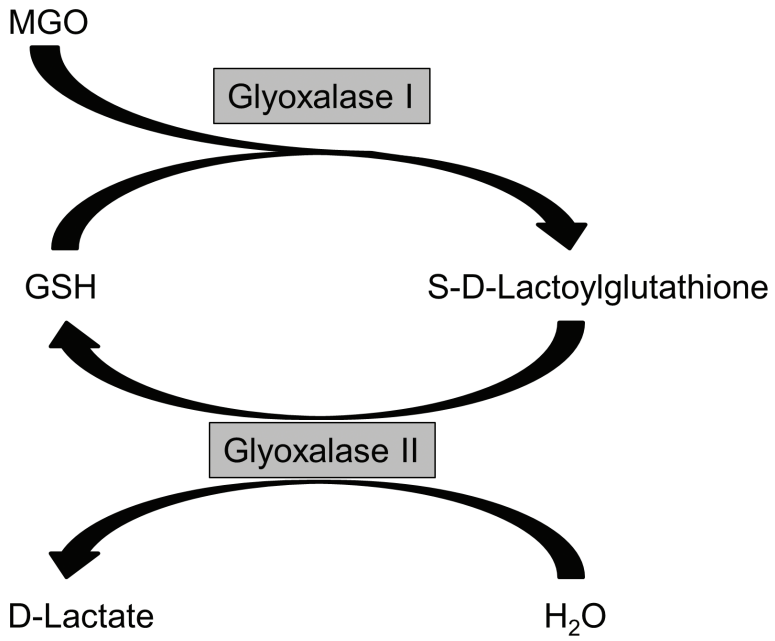


**Figure 1.** Glyoxalase system. Glyoxalase-I and glyoxalase-II comprise the glyoxalase system for detoxification of MGO. Glutathione is necessary as cofactor and is regenerated by Glo-II. Adapted from [43].

reaction with nucleotides, phospholipids, and proteins [50, 51], resulting in the formation of "advanced glycation end products (AGEs)" and reactive oxygen species (ROS) via AGEs or non-enzymatic reaction with hydrogen peroxide [52]. In this regard, MGO has shown to be involved in various inflammatory processes such as diabetes, aging, renal insufficiency, hypertension, or cancer [60–64].

Important MGO-derived AGEs are the non-fluorescent products 5-hydro-5-methylimidazolone (MG-H1) and tetrahydropyrimidine (THP) as well as the major fluorescent product, argpyrimidine [53, 54]. Other non-MGO-derived AGEs comprise $N^{\varepsilon}$-carboxymethyllysine (CML), pyrraline, or pentosidine [55]. The effects of AGEs have been allocated to their antagonistic receptor systems. The receptor for AGEs (RAGE) mediates generation of ROS, inflammation, angiogenesis, and proliferation [56, 57]. In contrast, AGE receptors (AGE-Rs), for instance, AGE-R1, are responsible for detoxification and clearance of AGEs [58]. Upon binding of AGEs to RAGE, various signal transduction pathways are activated. Recent studies showed involvement of the extracellular signal-regulated kinase 1/2 (ERK1/2), phosphoinositide 3-kinase (PI3-K)/protein kinase B (AKT), Janus kinase 2 (JAK2), and Rho GTPases, finally resulting in activation of NF-κB and production of pro-inflammatory cytokines (**Figure 3**) [59]. In addition, stimulation of RAGE resulted in activation of transforming growth factor (TGF-β) pathway and induced vascular endothelial growth factor (VEGF) overexpression [57].

In the last years, structure and genomic sequence of Glo-I was intensively analyzed. Glo-I is a dimer and consists in mammalian of two identical subunits with a molecular mass of 43–48 kDa [60]. Each subunit contains a zinc ion in its active center, whereas the apoenzyme remains catalytically inactive [45, 61]. The active center of Glo-I is localized between both monomers and comprises two structurally equivalent residues from each domain (Gln-33A, Glu-99A, His-126B, Glu-172B) and two water molecules indicating an octahedral arrangement [54, 62]. The protein sequence of Glo-I consists of 184 amino acids with post-translational modification of N-terminal Met [62].

Genomic analysis revealed three distinct phenotypes of Glo-I: GLO 1-1, GLO 1-2, and GLO 2-2 representing homo- and heterozygous expression of $GLO^1$ und $GLO^2$ [63, 64]. Gene locus of Glo-I is determined on chromosome six between centromere and human leukocyte antigen (HLA)-DR gene [65, 66]. Demographic studies showed higher distribution of $GLO^1$ in Alaska and lower $GLO^1$ allocation in southern and eastern Europe, America, Africa, and India [67].

Genetic sequencing identified association of distinct Glo-I phenotypes and Glo-I SNPs with diabetes [68], cardiovascular diseases [69], schizophrenia [70], autism [71, 72], anxiety [73], and cancer [74, 75]. These findings led to preliminary anti-tumor effects of Glo-I inhibition by siRNA or enzymatic inhibition in different cancer models [76–79]. In this regard, well-studied Glo-I inhibitors are S-ϱ-bromobenzylglutathione or S-ϱ-bromobenzyl-glutathione cyclopentyl diester [77, 80], methotrexate [81], indomethacin [82], troglitazone [83], and flavonoids [84, 85] showing anti-inflammatory and anti-tumor effects. Furthermore, an Glo-I inducer led to improved glycemic control and vascular function in 29 obese patients [86].

In a nutshell, Glo-I is responsible for detoxification of MGO and prevention of MGO-related formation of AGEs and ROS. Therefore, Glo-I and (R)AGE are involved in different pathophysiological inflammatory processes.

## 4. Glo-I and R(AGE) in fibrosis, cirrhosis, and NAFLD/NASH

### 4.1. Glo-I

To date, although Glo-I revealed an important role in inflammation, data about Glo-I in chronic liver disease remain preliminary. In an experimental approach of $CCl_4$-induced cirrhosis, Glo-I was analyzed *in vivo* and *in vitro* [87]. Wistar rats were treated with inhalative $CCl_4$ three times a week to induce early cirrhosis (without ascites) after 8 weeks or advanced cirrhosis (with ascites) after 12 weeks. Furthermore, primary liver cells from cirrhotic and noncirrhotic livers were isolated via portal vein perfusion and analysis of Glo-I was performed. Glo-I could be detected in HEP, HSC, and LSEC with highest expression on protein and mRNA levels in HEP. Furthermore, Glo-I expression was reduced in early and advanced cirrhosis in both whole liver and primary liver cells (**Figure 2A**). The reduction in Glo-I expression was greater with increasing severity of liver disease. Interestingly, the reduction of Glo-I was accompanied by an increase of MGO in cirrhosis (**Figure 2B**). This accumulation of MGO would lead to increased formation of AGEs and finally augment oxidative stress with ongoing inflammation in chronic liver disease [87]. So far, the reduction of Glo-I with consecutive increase of MGO would provide an explanation for perpetuating liver inflammation in advanced stages of liver disease.

Furthermore, modulation of Glo-I activity with the anti-inflammatory drug ethyl pyruvate (EP) was performed to analyze impact of Glo-I in initiation and progression of cirrhosis. EP is an $\alpha$-oxo-carbonic acid and ester of pyruvate. EP came in focus due to anti-inflammatory effects of pyruvate but low stability in aqueous solution [88]. Therefore, EP constitutes a more stable compound and exerts anti-inflammatory and protective effects in a lot of ROS-mediated models [89, 90]. Therefore, a possible molecular basis for the anti-inflammatory effects of EP was assumed to be the inhibition of specific Glo-I activity [91].

Since EP showed protective effects in acute liver failure [92–95] and development of fatty liver [96], effect of EP on activation of HSC, as it might occur in initial stadium of cirrhosis, was analyzed. Stimulation of HSC with LPS for 24 hours led to increased levels of $\alpha$-SMA, indicating activation of HSC and production of collagen deposit. This stimulation could be abrogated by modulation of Glo-I activity by means of EP (**Figure 2c**). Underlying mechanisms involve stimulation of Nrf2 as well as reduction of NF-κB and ERK/pERK by EP. Additional *in vivo* experiments revealed reduced collagen deposit in Wistar rats that were treated with $CCl_4$ for 12 weeks and i.p. EP [87]. Furthermore, EP-treated rats revealed significantly less Sirius red staining and consequently less fibrosis compared with controls receiving saline (**Figure 2D**).

Indeed, anti-inflammatory treatment of several diseases with EP might be a promising future clinical approach. However, EP was analyzed in a clinical trial (phase-II multicenter double-blind placebo-controlled study) in high-risk patients undergoing cardiac surgery with cardiopulmonary bypass. This trial was performed in 13 US hospitals including patients with

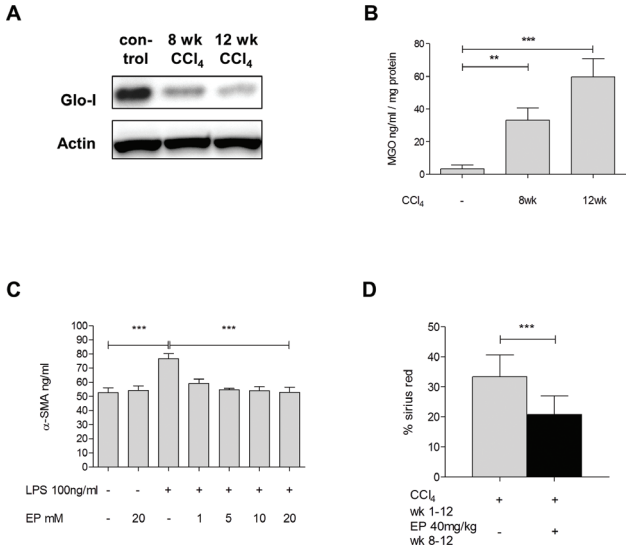


**Figure 2.** Glyoxalase-I in $CCl_4$-induced cirrhosis. **(A)**, Glo-I expression was reduced in early (8 week $CCl_4$-treatment) and advanced (12 week $CCl_4$-treatment) cirrhosis in Western blot. Wistar rats were treated three times per week with inhalative $CCl_4$ for induction of cirrhosis. **(B)**, MGO levels were significantly elevated in cirrhosis, indicated by ELISA-analysis. **(C)**, treatment of stellate cells (HSC) for 24 hours with LPS revealed increased production of $\alpha$-SMA. Cotreatment with Glo-I modulator ethyl pyruvate (EP) abolished the LPS-induced effects. **(D)**, Wistar rats were treated with $CCl_4$ and i.p. EP or saline from week 8 to 12. Sirius red staining indicated significantly less fibrosis in EP-treated animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Adapted from [87].

a Parsonnet risk score > 15 undergoing coronary artery bypass graft and/or cardiac valvular surgery with cardiopulmonary bypass. 102 subjects received either placebo (53) or 7.500 mg (90 mg/kg) EP (49) intravenously followed by five more doses every 6 hours. The primary endpoint was a combination of death, prolonged mechanical ventilation, renal failure, or need of vasoconstrictors. No statistically significant differences were observed between groups with regard to clinical parameters or markers of systemic inflammation [97]. Despite these disappointing results in the first clinical trial, it should be kept in mind that underlying molecular mechanisms in cardiac surgery with cardiopulmonary bypass are complex and at least partly different from ROS models showing protective effects of EP. Another clinical study design, for example, liver fibrosis, pancreatitis, septic shock, might be more promising for this interesting agent.

In summary, targeting Glo-I with EP in cirrhosis revealed an innovative therapeutic target. Nevertheless, further research needs to confirm the aforementioned results in further animal experiments and clinical trials.

### 4.2. AGEs

In contrast to straightforward evidence of Glo-I in chronic liver disease, several groups analyzed AGEs in liver fibrosis, cirrhosis, and NASH. In cirrhotic patients, limited amount of methylglyoxal-modified proteins were found to be elevated compared to controls [98].

Another study revealed increased levels of CML-AGEs in blood plasma of cirrhotic patients. Also, CML levels correlated with severity of disease [99]. Additional studies confirmed the observations of increased CML levels in fibrosis and cirrhosis [100, 101]. These clinical findings were supported by laboratory analysis: *in vitro* treatment of HSC with AGEs resulted in enhanced production of oxidative stress providing evidence of AGEs-involvement in fibrosis [102]. Conversely, oxidative stress was found to elevate levels of CML in rats [103] and incubation of HSC with AGEs led to elevation of $\alpha$-SMA, TGF-$\beta$, and collagen-I [104]. In addition, treatment of rat hepatocyte cultures with AGEs reduced cell viability [105]. In an interesting translational study, CML-AGEs were positively correlated with liver stiffness in patients with chronic hepatitis C. *In vitro* data showed in this study enhanced cell proliferation of HSC treated with BSA-AGEs (CML) and increased production of $\alpha$-SMA. In contrast, in another study, intraperitoneal administration of AGE-rat serum albumin (CML) revealed increased levels of $\alpha$-SMA and fibrosis in a model of bile duct ligation [106]. Furthermore, AGEs were found to induce autophagy which subsequently contributes to the fibrosis in patients with chronic hepatitis C [107]. The finding that AGEs were elevated in fibrosis and treatment with AGEs-induced fibrosis led to an interventional approach targeting AGEs to prevent induction of chronic liver disease. Indeed, inhibition of CML resulted in attenuation of CML-induced levels of $\alpha$-SMA and ROS in HSC [108].

Another model to study fibrosis belongs to metabolic liver diseases: induction of NASH by means of methionine choline deficient diet (MCD). Therefore, hepatic steatosis induced by MCD showed accumulation of CML, and CML was associated with grade of hepatic inflammation and gene expression of inflammatory markers (PAI-1, IL-8, and CRP) [109]. AGEs have also been shown to be involved in etiology of insulin resistance and diabetes [110], and rats fed with a diet rich in AGEs showed elevated oxidative stress and hepatic inflammation leading to NASH [111]. In addition, high dietary AGEs increased hepatic AGEs levels and induced liver injury, inflammation, and liver fibrosis via oxidative stress in activated HSC [112]. Another interesting study investigated the underlying mechanism of AGEs-crosstalk in NASH. AGEs induced NOX2 leading to downregulation of Sirt1/Timp3 and finally resulting in activation of TNF-$\alpha$ converting enzyme and inflammation. These pro-inflammatory cascades finally led to NASH and fibrosis [113]. Interventional studies on AGEs reduction in NASH also revealed promising results. The flavonoid curcumin eliminated the inflammatory effects of AGEs in HSC by interrupting leptin signaling and activating transcription factor Nrf2, which led to the elevation of cellular glutathione levels and the attenuation of oxidative stress [114]. In addition, curcumin decreased activation and proliferation of HSC by AGEs and induced gene expression of AGE-clearing receptor AGE-R1 [115]. The use of the LDL-lowering drug atorvastatin [116] or combination therapy of telmisartan and nateglinide [117] also decreased levels of AGEs in patients with NASH and dyslipidemia, leading to improvement of steatosis, nonalcoholic fatty liver disease activity score, and amelioration of insulin resistance. Another study evaluated effects of aqueous extracts from Solanum nigrum (AESN). AESN could reduce the AGE-induced expression of collagen-II, MMP-2, and $\alpha$-SMA in HSC. Also, AESN improved insulin resistance and hyperinsulinemia and downregulated lipogenesis, finally preventing fibrosis [118].

Having the auspicious and conclusive effects of AGEs-lowering drugs in fibrosis in mind, it should be noted that mainly CML-AGEs were investigated. Therefore, it should be considered

that CML-AGEs are rarely produced via reaction of MGO but are rather formed in lipoxidation and glycoxidation independent of MGO [119].

### 4.3. RAGE

The pattern recognition receptor RAGE belongs to the immunoglobulin superfamily with a molecular mass of 47–55 kDa. RAGE expression is stimulated under inflammatory conditions such as diabetes, cardiovascular diseases, or cancer [120]. RAGE has been shown to be activated by MGO- and non-MGO-derived AGEs as well as multiple ligands. Binding to RAGE results in activation of transcription factors, such as NF-κB [121], leading to the release of pro-inflammatory cytokines.

Indeed, several studies revealed participation of RAGE in fibrosis: Upon stimulation with AGE-rat serum albumin containing mainly CML, levels of RAGE, α-SMA, hydroxyproline, and Sirius red were elevated in a fibrosis model of bile duct ligation (BDL) [106, 122]. Interestingly, RAGE was found to be predominantly expressed in HSC. RAGE was stimulated in HSC during transformation to myofibroblasts, and RAGE was colocalized with α-SMA and induced by TGF-β. In addition, RAGE was expressed in filopodial membranes of myofibroblasts suggesting a role of RAGE in spreading and migration of activated HSC in fibrogenesis [123]. Further analysis provided evidence for crosstalk of RAGE and TGF-β: AGEs-induced upregulation of RAGE induced TGF-β, TNF-α, and IL-8. Interestingly, RAGE also stimulated anti-inflammatory cytokines IL-2 and IL-4 indicating a negative feedback mechanism and inhibitory crosstalk between TGF-β and RAGE [124]. In the next step, effect of RAGE inhibition on inflammation and fibrosis was discovered. First, curcumin was found to reduce, besides its AGEs-lowering effects, the gene expression of RAGE via elevation of PPAR-γ [125]. Furthermore, RAGE expression was diminished by means of RAGE siRNA in primary rat HSC resulting in downregulation of IL-6, TNF-α, and TGF-β [126]. In a following *in vivo* study, effects of repetitive RAGE siRNA in an olive oil model of fibrosis were analyzed. RAGE siRNA was injected twice weekly in the tail vein of Sprague-Dawley rats. After 6 weeks, reduced expressions of RAGE, TNF-α, IL-6, extracellular matrix, hyaluronic acid, and procollagen III were found. Also, activation of HSC and NF-κB was reduced in siRNA-treated animals attenuating the initiation and progression of fibrosis [127]. Additional studies revealed protective effects of anti-RAGE antibodies in BDL-induced acute liver injury [128, 129].

Growing evidence for implication of RAGE in fibrosis was found in NASH. Methionine choline deficient (MCD) diet caused steatosis and increased RAGE, inflammation, and fibrosis [112]. Recently, fatty acids stimulated CML accumulation and subsequently elicited RAGE induction [109]. Another group found upregulation of RAGE in the liver of aged mice with consecutive elevated oxidative stress shown by analysis of malondialdehyde. Blocking of RAGE by anti-RAGE-antibody revealed in this study prolonged survival of animals [130].

In a nutshell, various studies confirmed implication of Glo-I and (R)AGE in inflammatory liver disease and fibrosis. Especially targeting Glo-I in cirrhosis highlighted the meaning of MGO-induced liver damage and offers new therapeutic opportunities. Nevertheless, further research in this topic will uncover the exact role of Glo-I in chronic liver disease and possible translation to clinical approach (see **Figure 3**).

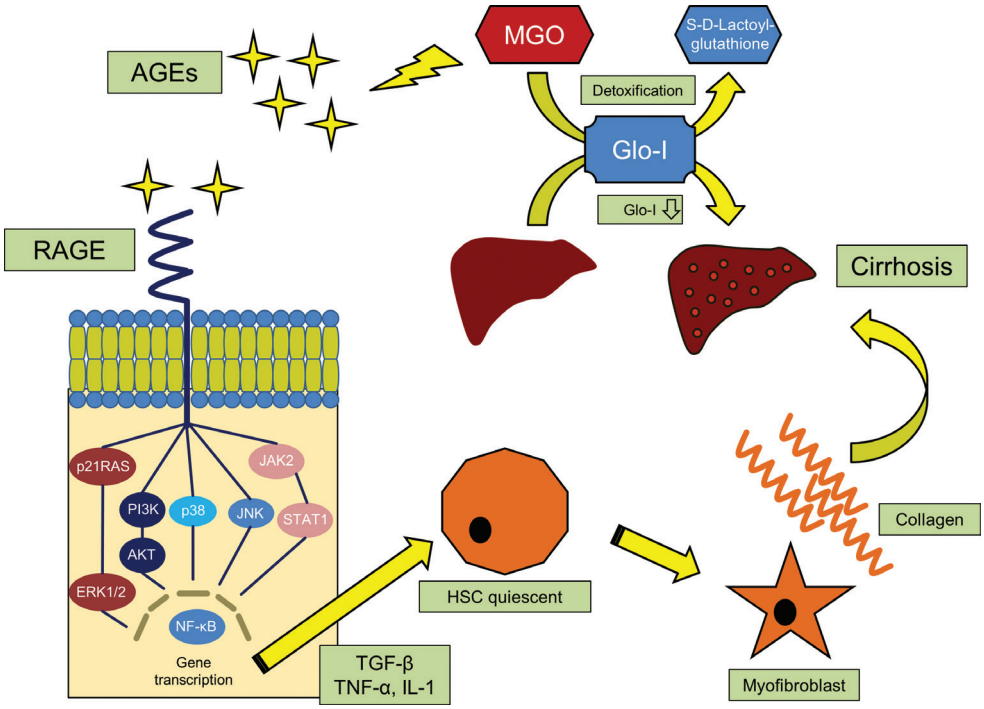


**Figure 3.** Impact of Glo-I and (R)AGE in cirrhosis. MGO reacts with proteins, nucleotides, and lipids leading to formation of AGEs. AGEs bind to RAGE and activate several signal pathways (including MAPK (ERK1/2, p38, JNK), PI3-K/AKT, and JAK2/STAT1), finally leading to activation of NF-κB. In a consequence, the induced production of TGF-β and pro-inflammatory cytokines activate quiescent stellate cells. HSC transform to myofibroblasts and produce pro-fibrotic factors and collagen. The collagen deposition in the liver will lead to fibrosis and finally cirrhosis. Reduction of Glo-I will perpetuate both, initiation and progression of cirrhosis due to increase of MGO and a vicious circle of disease. MGO: methylglyoxal, AGEs: advanced glycation end products, RAGE: receptor for advanced glycation end products, Glo-I: glyoxalase-I, HSC: hepatic stellate cells, MAPK: mitogen-activated protein kinase, PI3-K: phosphoinositide 3-kinase, AKT: protein kinase B, JAK2: Janus kinase 2, STAT1: signal transducer and activator of transcription-1, JNK: c-Jun N-terminal kinase, and NF-κB: nuclear factor-κB.

## Abbreviations

| | |
|---|---|
| AGEs | advanced glycation end products |
| AKT | protein kinase B |
| EP | ethyl pyruvate |
| ET-1 | endothelin-1 |
| Glo-I | glyoxalase-I |
| Glo-II | glyoxalase-II |
| GSH | L-glutathione |
| HCC | hepatocellular carcinoma |

| | |
|---|---|
| HEP | hepatocytes |
| HSC | hepatic stellate cells |
| JAK2 | Janus kinase 2 |
| JNK | c-Jun N-terminal kinase |
| KC | Kupffer cells |
| LSEC | liver sinusoidal endothelial cells |
| MAPK | mitogen-activated protein kinase |
| MCD | methionine choline deficient diet |
| MG-H1 | 5-hydro-5-methylimidazolone |
| MGO | methylglyoxal |
| NAFLD/NASH | non-alcoholic fatty liver disease/steatohepatitis |
| NF-κB | nuclear factor-κB |
| NO | nitric oxide |
| PI3-K | phosphoinositide 3-kinase |
| RAGE | receptor for advanced glycation end products |
| sRAGE | soluble form of RAGE |
| ROS | reactive oxygen species |
| STAT1 | signal transducer and activator of transcription-1 |
| TGF-β | transforming growth factor beta |
| THP | tetrahydropyrimidine |

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Chapter 12

# Regenerative Medicine in Liver Cirrhosis: Promises and Pitfalls

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Additional information is available at the end of the chapter

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**Abstract**

Liver cirrhosis is irreversible and mostly ends up with complete loss of liver function/end-stage liver failure, and the only proven treatment is liver transplantation. Scarcity of donor, high cost, lifelong immunosuppression, and surgical complications are the major issues associated with liver transplantation and these urge to look for alternate therapeutic approaches. Advancements in the field of regenerative medicine are arising hope for the treatment of liver cirrhosis. This chapter deals with the scope of liver regenerative medicine in the treatment of liver cirrhosis. Review of the literature showed that liver regenerative medicine no doubt holds great promises and added a lot of hope to the cure of liver diseases. Primarily, cell-based therapies had shown great potential to treat liver cirrhosis. Successful clinical human trials further strengthen their significance in the field. However, recent trends in liver regenerative medicine are focusing on the development of tissue engineering leading to generation of the whole organ. Despite advantages, liver regenerative medicine has several limitations and sometimes been over-optimistically interpreted. In conclusion, the current scenario advocates to conduct more preclinical and clinical trials to effectively replace liver transplantation with liver regenerative medicine to treat liver diseases.

**Keywords:** regenerative medicine, stem cells, hepatocytes, tissue engineering

## 1. Introduction

Liver is one of the largest and most important metabolic organs in the human body with considerable regeneration capacity. However, in prolonged hepatic injuries, the regeneration capacity of hepatocytes times out and a cascade of life-threatening complications is initiated

leading to liver cirrhosis. Liver cirrhosis is irreversible and mostly ends up with complete loss of liver function/end-stage liver failure. End-stage liver failure with high rates of morbidity and mortality poses a significant threat to human health as well as economy throughout the world [1]. As current pharmacological treatments are inefficient to reverse this loss, liver transplantation is the only effective lifesaving option. Since the first liver transplantation in 1963, the number of cases requiring transplantation are considerably increasing with the passage of time. Despite the success of liver transplantation, there is a gap between demand and supply. Only 30–50% of annual liver donation desires are fulfilled and at least about 15% patients die while being on the waiting list [2, 3]. Besides scarcity of liver donors, high cost, postoperative graft rejection, and long-term immune-suppression are few more serious constraints associated with liver transplant [4]. Therefore, it is crucial to look for effective and operative alternate approaches of liver transplantation.

Advancements in the field of regenerative medicine open up new horizons and arising hope in the treatment of irreversibly damaged liver cirrhosis. Liver regenerative medicine mainly emphasizes on the establishment of new therapies to either functionally restore the chronically damaged liver tissue or to develop the entire new organ [5]. Elucidation of cellular and molecular mechanisms during the last couple of decades in the field of hepatic organogenesis and regeneration provides milestones in the development of liver regenerative medicine. Moreover, compared to current operative therapies, it is less invasive, is less expensive, and avoids the problem of shortage of donors, immune rejection, and other similar complications. Ideally, liver regenerative medicine seems an ultimate solution for liver cirrhosis.

Liver regenerative medicine uses two key approaches based on cell therapy and tissue/organ engineering. Cell-based therapy is defined as the transplantation of cells from different sources with or without differentiation to improve liver function [6]. Transplantation of mature hepatocytes and liver stem/progenitor cells (LSPCs) from allogeneic sources is already in clinical trials. However, current research is intended to overcome the problem of immune rejection associated with allogeneic sources and focuses on therapies based on generation of autologous hepatocytes from MSCs and induced pluripotent stem cells (iPSCs) [5]. Elucidation of cell type, which can be successfully differentiated into functional and transplantable hepatocytes or liver progenitor cells, is another major task under study [7]. Furthermore, researchers are trying to refine protocols for proliferation, differentiation, and storage of these cells to have them in plenty and always ready to be transplanted.

Second strategy mainly covers the area of liver tissue/organ engineering, engraftment, and monitoring in patients. Ongoing therapeutic approaches in tissue engineering include implantable constructs of hepatic tissues and whole organ. For the construction of hepatic tissues, natural and synthetic bioactive scaffolds are designed [5]. Nanotechnology and microchip devices are contributing a lot in this lane. Moreover, whole organ engineering is also in great focus to escape end-stage liver diseases. However, determination of ideal cell types, cell volume, and optimal seeding techniques is yet to be discovered [8, 9].

This chapter deals with the scope of liver regenerative medicine in the treatment of liver cirrhosis. Different operative and proposed therapies along with their pros and cons are the major focus of this section and are reviewed in detail.

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## 2. Hepatic organogenesis

Zygote is the only totipotent structure that leads to the development of blastocyst. Blastocyst carries both embryonic and extraembryonic (inner cell mass) cell population. Inner cell mass (ICM) forms three germ layers: exoderm, mesoderm, and endoderm. Embryonic liver develops from the endodermal layer during ventral foregut closure in the midgut [10]. Cells residing in the hepatic bud are bipotent and are called hepatoblasts. Hepatoblasts are columnar in shape, release $\alpha$-fetoprotein, and differentiate into mature hepatocytes and cholangiocytes [11].

Wingless type (wnt) signaling pathway, together with activin-A, plays a crucial role in the establishment of endoderm during primitive streak formation and differentiation of liver precursor cells toward hepatoblasts [12, 13]. Other key factors involved in hepatic fate determination are fibroblast growth factors (FGFs) released from cardiac mesoderm and bone morphogenetic proteins (BMPs) released by septum transversum mesenchyme [3]. Furthermore, oncostatin M and hepatocyte growth factor (HGF) control the differentiation of hepatoblasts toward hepatocytes [14], whereas Jagged-Notch signaling pathway is responsible for the development of cholangiocytes [15].

Gradually, as the liver development proceeds toward the final stages of maturation, hepatoblast number reduces markedly. Liver becomes populated with mature and unipotent hepatocytes and cholangiocytes. The remainder resident cells of liver, that is, Kupffer cells, stellate cells, and endothelium, are mesodermal in origin. Majority of the liver functions are performed by hepatocytes. On the onset of any hepatic insult, adult liver cells undergo apoptosis that calls for the replacement of lost cells or in other words liver regeneration. The schematic diagram of liver organogenesis from endodermal layer along with important molecular signaling pathways involved in activation or suppression of each step has been represented in **Figure 1**.

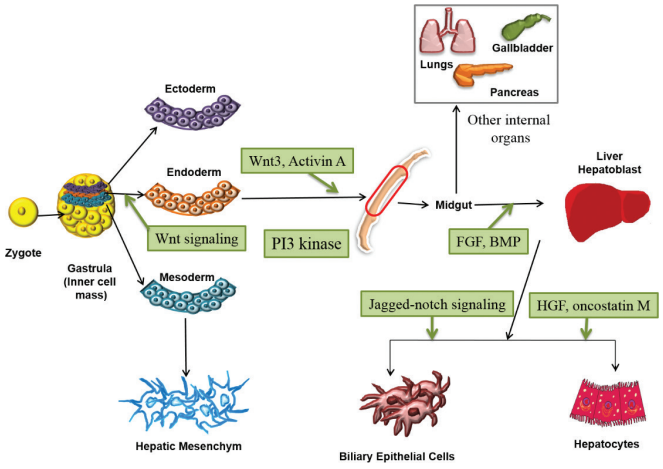


**Figure 1.** Schematic diagram of liver organogenesis. Molecular signals involved in the activation of each stage are indicated in the boxes occuring at various steps of liver organogenesis.

## 3. Liver regeneration

Elucidation of the cellular and molecular mechanisms involved in liver regeneration provides vital scientific grounds for liver regenerative medicine. Depending upon the origin of liver damage, different kinds of repair mechanisms are operative [16]. Various surgical and toxin-mediated injury models for liver regeneration have been established so far. One of the established and utterly studied model of regeneration is rodent partial hepatectomy [17]. In partial hepatectomy model, liver can regenerate to its normal size in 3–10 days even if two-thirds of its mass is surgically removed. A fine coordination of cellular and molecular events occurs in the regeneration process of partial hepatectomy. Robust hepatocyte replication followed by hypertrophy has been revealed as an underlying cellular mechanism in partial hepatectomy recovery. This vigorous change in hepatocytes is also accompanied by alteration of gene expression patterns, instigation of transcription factors, and release of growth signals. More than 100 genes are activated in an early response manner. At least 40% of these early response genes are activated by interleukin-6 (IL-6) signaling which itself is activated by tumor necrosis factor-$\alpha$ (TNF-$\alpha$)-mediated NF$\kappa$B (nuclear factor kappa-B) activation [18, 19]. The recovery of liver mass and function of living donor and recipient of liver transplantation in humans seems to adopt a similar track.

Besides utilizing mature hepatocytes for liver regeneration, another likely approach is the use of liver progenitor cells (LSPCs). They are capable of converting into different cell lines found in liver, that is, hepatocytes, oval cells, and stellate cells [20]. LSPCs got experimental and clinical support when they were overproliferated in case of induced liver injury by acetaminophen and slowly proliferated in case of liver cirrhosis [21, 22]. At present, the main focus is on the regenerative capacity of LSPCs when hepatocytes run out of their regenerative potential. LSPCs are also proved potential progenitor cells of biliary epithelium in vitro, but no specific LSPC markers are identified as yet. It seems that LSPCs are driven by the activation of certain genes and the combination of growth factors. Crucially important genes include Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) and the cytokine tumor necrosis factor-like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor (TNF) superfamily [23]. Some other mitogenic factors also play a crucial role, for example, HGF, epidermal growth factor (EGF), TGF-$\alpha$, and fibroblast growth factors 1 and 2 (FGF1 and FGF2) [24]. However, there is lack of evidence pertaining to in vivo differentiation of LSPCs into hepatocytes. The articles published in 2014 used different methodologies to trace the fate of liver progenitor cells. They utterly rejected the concept of regenerative capability of LSPCs into hepatocytes. Besides, despite lack of proof of the in vivo hepatogenic differentiation of LSPCs, they surely can give rise to hepatocyte-like cells in vitro [20]. Research in this arena is ongoing and there is a probability that even in mice a part for oval cells/LSPCs in regeneration will be found.

Third major concept in liver regeneration is through extrahepatic cells that is hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) derived from bone marrow. HSC and MSC from bone marrow reach the liver via blood circulation. These HSCs and MSCs can populate the liver after hepatogenic differentiation [25]. It is proposed that these bone marrow-derived stem cells are not directly converted into hepatocytes rather they first mix

with resident liver cells and then participate in liver repopulation [26]. It has also been suggested that MSCs with multilineage differentiation potential provide a great variety of cells for nonhematopoietic tissues like liver tissues [27]. Though they are highly heterogeneous in nature, only a little fraction of it contributes to liver regeneration [28]. It is notable that bone marrow cells take part in the regeneration of liver endothelium. Twenty percent of the liver endothelial cells are made by the bone marrow-derived endothelial cells [29]. There is a need of concerning involvement of bone marrow-derived stem cells in liver parenchyma regeneration, for designing the methods for cellular therapy of liver disease [16].

## 4. Cell-based therapies for regeneration of liver cirrhosis

Cell-based therapies are the oldest and most efficient method to regenerate damaged liver. Effective engraftment and proliferation of donor cells in the recipient liver are the main issues of concern for liver regeneration through cell-based therapy. Depending on the donor source, cells can be of autologous [30], allogeneic, or syngeneic nature [31]. The cells are injected into the recipient through portal vein, peripheral vein [30], and intraspleenic [32] or intraperitoneal route. To enhance the transplantation efficiency, conditioning of recipient liver with partial hepatectomy [33, 34], liver irradiation [35, 36], or portal embolization [37] has been recently proposed. Broadly, cells are categorized into two main categories; stem cells and mature hepatocytes are the potential cell-based therapies adapted to date in the cure and regeneration of liver cirrhosis [5]. The roles of these cell-based therapies are shown in **Figure 2** and are discussed one by one in detail in the following section.

### 4.1. Hepatocytes and liver regeneration

Liver is chiefly composed of hepatocytes. Hepatocyte proliferation plays a distinctive role in liver regeneration under both acute and chronic injury conditions. The unique characteristic

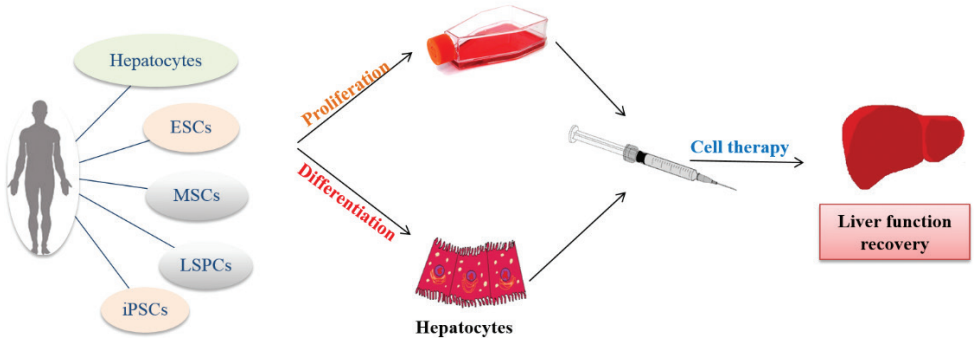


**Figure 2.** Different types of cells and their mode of application for cell-based therapies of liver cirrhosis. Different types of cells isolated from humans and being used in liver regeneration are shown on the left side of the figure. Each of the cell type has been injected and has recovered liver functions either through only in vitro proliferation (hepatocytes), via differentiation toward hepatocytes (ESCs and iPSCs) or through both (MSCs, LSPCs).

of hepatocytes to proliferate under stress conditions makes them ideal cell type for cell-based therapies. Primary hepatocytes were the very first type of cells to be used for cell-based therapy of liver. Isolated hepatocytes are infused either directly into the liver or into the spleen from where they can migrate to and settle down in the liver. The hepatocyte transplantation has shown to considerably improve the hepatic functions even in end-stage liver failure [38]. Typically, hepatocytes are harvested from the livers that are not suitable for transplantation [39]. However, due to problem of immune rejection, it was also tried to isolate hepatocyte from patient's biopsies [40].

Although primary hepatocytes are ideal for use in liver regeneration, this approach is prone to certain limiting factors. Inadequate supply of the required cells, slow in vitro proliferation rate [18], dedifferentiation within 72 hours of culturing [41], susceptibility to freeze-thaw damage, and loss of certain characteristic features in culture conditions are major obstacles that hinder the utilization of these cells for liver regeneration [38]. The isolated primary hepatocytes are of low quantitative value, and an autologous isolation of this cell population involves patients' inconvenience. Typically, hepatocytes are harvested from the livers that are not suitable for transplantation, so the quantitative and qualitative values of obtained cells vary considerably. All of these constraints have played a pivotal role in shifting focus toward alternate cell-based therapies.

### 4.2. Stem cells in liver regeneration

With the therapeutic focus being set on the establishment of personalized medicine and the replacement or regeneration of damaged tissue, stem cell-based therapies may provide a strong platform. The properties of indefinite cell division and differentiation potential into other cell types make the stem cells an ideal choice for cure and regeneration of liver cirrhosis. Another important property of stem cells is their ability to create and provide a favorable environment for growth of primary hepatocytes and/or hepatocyte-like cells [5]. Coculturing MSCs with primary hepatocytes results in their improved viability and function by providing structural and paracrine trophic support [41–43]. Moreover, stem cell therapy holds great potential especially in the cure of inherited liver diseases, where, together with gene therapy, it may correct metabolic disorders permanently without even using immunosuppressive drugs [5]. Chiefly, two approaches of stem cell-based liver regeneration are in practice either their direct injection or in vitro differentiation toward hepatocyte-like cells and transplantation.

Some types of stem cells show efficient growth in vitro, could be a rich pool to supply hepatocytes/precursor cells, and thus be used largely for transplantation. If the wide availability of human hepatocytes is made possible, this could be a major breakthrough in the treatment of various liver diseases. However, the research work debating good capacity stem cell therapy lack in reproducibility evidence or some of these even have been overoptimistically interpreted. Another important milestone is to decide on the preference of stem and precursor cell types. It is a difficult task to compare different cell types with respect to their reported capacity of differentiation toward hepatocytes [44]. We therefore discuss the possibilities these cell therapies offer one by one, along with the limitations which are making these feats harder to achieve.

*4.2.1. Embryonic stem cells and hepatocyte generation*

Differentiation of cultured embryonic stem cells toward hepatocyte-like cells in vitro appears to be the most studied model of mature hepatocyte generation. In mouse models of liver injury, hepatocyte-like cells not only recover the liver by proliferation but also provide trophic factors that assist the endogenous hepatic regenerative capability [45]. Human ESCs efficiently form embryoid bodies in suspension cultures forming three germ layers [46]. Hepatocyte isolation from this heterogeneous cell population is very difficult, suggesting endoderm enrichment to be a practical option with maximum hepatocyte yield.

A directional differentiation strategy for the generation of functional hepatocytes from embryonic stem cells involves sequential supplementation of various molecular factors (growth factors and cytokines necessary for development of human embryonic liver)-enriched growth medium. The molecular factors involved in early embryonic differentiation such as fibroblast growth factor (FGF2/4), bone morphogenetic protein (BMP2/4), activin A and Wnt3 can be used for endoderm enrichment from cultivated embryoid bodies [44, 46]. FGF2/4 stimulates the development of hepatoblasts from cultured ESCs and the generation of mature hepatocytes, whereas HGF plays a supportive role in hepatocyte generation from hepatoblasts. Dexamethasone (glucocorticoid hormone) induces the production of adult hepatocyte-specific proteins. This strategy ensures an 80–90% hepatocyte yield. Recently, Wang et al. established a polymer-modified nanoparticle-based sustained delivery system for growth factors to direct stem cell differentiation into hepatocytes [47]. Their approach can help to overcome the limitations linked with current models and make sure efficient delivery of growth factors to improve ESC differentiation toward a hepatocyte-like lineage.

The final and most important step in this strategy involves isolation of absolute hepatocyte population from a heterogeneous mixture containing other hepatic precursors and immature hepatocytes. Basma et al. used asialoglycoprotein receptor ASGPR1 (hepatocyte-specific cell surface marker) expression based sorting to enrich the pure hepatocyte populations [48]. To enhance the isolation efficiency of hepatocytes based on ASGPR1, fluorescent-labeled or magnet-coated antibodies are further proposed [49]. However, further research is required to be performed to isolate definitive hepatocyte population or to obtain a relatively absolute ratio of hepatocytes from ESCs [50].

Despite their success stories, there are a number of ethical issues concerning the use of human ESCs in liver regenerative medicine [50]. Furthermore, pluripotency of these cells is very difficult to handle leading to an uncontrolled regenerative potential. Above all, putative tumorigenicity associated with transplantation of ESCs proves to be an additional barrier for their clinical application [49–50].

*4.2.2. Bone marrow stem cells (BMSCs)*

In bone marrow, three different pluripotent cell populations, that is hematopoietic stem cells (HSCs), MSCs, and multipotent adult progenitor cells (MAPCs)/endothelial progenitor cells (EPCs), are present [51]. Peripheral blood, umbilical cord blood, and synovial fluid are additional sources of HSCs and MSCs. HSCs and MSCs can be advantageous cell sources for liver

regeneration as compared to hepatocytes since they can be obtained relatively easily from blood and bone marrow of live donors. Since BMSCs are immune-modulators, a reduced chance of graft rejection is an additional property of these stem cells [47, 51]. In clinical trials, patients with autologous BMSC ($CD34^+$ cell) transplantation had no procedure-related complications and showed improved quality of life [30]. MSCs have proven reliable for treatment of liver cirrhosis in phase I and phase II clinical trials as shown in **Table 1**.

| Cell source | Liver cirrhosis | No. of patients | Administration route | Follow-up period | Outcomes/clinical significance | References |
|---|---|---|---|---|---|---|
| Hepatocytes (autologous) | Liver cirrhosis | 9 | intraportal | 10 months in only one patient | Longer survival | [40] |
| $EpCAM^+$ Fetal liver-SCs | Advanced cirrhosis | 2 | hepatic artery | 12 months | Biochemical and clinical improvement | [74] |
| | End-stage liver cirrhosis | 25 | hepatic artery | 6 months | Improved liver function and MELD score | [32] |
| BM-MSCs | Decompensated liver cirrhosis | 4 | peripheral vein | 12 months | Well-tolerated and safe procedure; improved liver function | [75] |
| | post-HCV liver cirrhosis | 20 | intrasplenic | 6 months | Decreased TBIL, AST, ALT, PT; improved ALB, PC, PT, INR | [76] |
| Autologous BM-MSCs | Alcoholic cirrhosis | 11 | hepatic artery | 12 months | No significant side effects; histological improvement; improved CP score | [77] |
| | Liver cirrhosis | 9 | peripheral vein | 6 months | No major adverse effects; improved ALB, CP scores | [78] |
| BM-MSCs (Differentiated *vs* undifferentiated) | post-HCV liver cirrhosis | 10: control 15: treated | intravenous | 6 months | Improved MELD score, BIL, ALB, and PC | [79] |
| UC-MSCs | Primary biliary cirrhosis | 7 | peripheral vein | 12 months | No obvious side effects; decreased serum ALP and GGT | [80] |
| | Post-HBV decompensated liver cirrhosis | 15: control 30: treated | intravenous | 12 months | No significant side effects; improved liver function and MELD score; reduced ascites | [81] |

| Cell source | Liver cirrhosis | No. of patients | Administration route | Follow-up period | Outcomes/clinical significance | References |
|---|---|---|---|---|---|---|
| Autologous MSCs from iliac crest | Decompensated cirrhosis | 12: control 15: treated | peripheral vein | 12 months | No beneficial effect | [82] |
| | End-stage liver disease | 8 | peripheral or portal vein | 6 months | No adverse effects; improved MELD and liver function | [83] |
| Allogenic MSCs | Autoimmune disease-induced liver cirrhosis | 26 | peripheral vein | 24 months | No obvious side effects; improved MELD and liver function | [84] |
| G-CSF mobilization of CD 34$^+$ BMSCs | Severe liver cirrhosis | 40: controls 8: treated | subcutaneous | 8 months | No adverse events; improved MELD score | [85] |
| | Alcoholic cirrhosis | 11: control 13: treated | subcutaneous | 3 months | Effective CD34$^+$ cells mobilization; increased HGF; induced hepatocyte proliferation | [86] |
| | Liver cirrhosis | 18 | subcutaneous | 3 weeks | No severe adverse events; no liver function significant modification | [87] |
| Autologous G-CSF-mobilized cultured CD34$^+$ BMSCs | Alcoholic liver cirrhosis | 9 | hepatic artery | 3 months | No side effects; improved BIL, ALT, AST, CP score and ascites | [88] |
| PBMCs from G-CSF mobilized PB | Decompensated liver cirrhosis | 20: control 20: treated | | 6 months | No major adverse effects; improved liver function | [89] |

EpCAM: Epithelial cell adhesion molecule; GGT: γ-glutamyl transferase; ALT: Alanine aminotransferase; TBIL: Total bilirubin; AST: Aspartate aminotransferase; CP: Child-Pugh; HGF: Hepatocyte growth factor; HCV: Hepatitis C virus; PT: Prothrombin time; ALB; Albumin; PC: Platelet count; INR: International normalized ratio; MELD: Model for end-stage liver diseases; ALP: Alkaline phosphatase; UC-MSC: Umbilical cord blood-mesenchymal stem cells; G-CSF: Granulocyte-colony-stimulating factor; BM-MSCs: Bone marrow-mesenchymal stem cells.

**Table 1.** Clinical trials of cell-based therapies along with their route of administration, follow-up, and outcomes.

Hematopoietic stem cells originating from bone marrow are efficient stem cell population that migrates to the site of injury and participate in the repopulation of damaged tissue. In liver regeneration, this stem cell population is postulated to contribute based on the cell fusion capability of the BMSCs [52, 53] rather than cellular differentiation. In murine hepatectomy models, BMSCs were found to fuse with hepatocytes, and the resultant hybrid cells were shown to be responsible for triggering proficient liver regenerative reaction [54]. Therapeutic mechanisms of MSCs are reported to be more clear as compared to those of HSCs. MSCs not only reduce

inflammation and fibrosis but they also increase liver regenerative response in a much rapid manner than HSCs [55]. CD34 is reported to be an efficient cellular marker for the isolation of HSCs [30]. However, these cells have showed profibrogenic potential in some cases [56].

Despite wide use in preclinical setting and clinical trials, the BMSCs have to be evaluated extensively for their potential role in liver regeneration before being applied to the wide clinical utilization. Tumorigenicity of MSCs is another constraint that needs to be considered while using this stem cell population in clinical application [57].

*4.2.3. Adipose-derived stem cells (ADSCs)*

Adipose tissue is another source of MSCs used for hepatic regeneration. ADSCs seem to be pluripotent and have the potential to differentiate into cells of multiple germ lines such as bone, nerve, heart, and adipose tissue. These cells are advantageous over BMSCs because of their higher in vitro proliferation activity and differentiation potential [58]. The sufficient availability of adipose tissue from most patients with no substantial defects renders ADSCs an efficient alternative source of stem cells for liver regeneration [59]. Differentiation of ADSCs into functional hepatocytes involves activation of Wnt/beta-catenin signaling through glycogen synthase kinase 3 inhibitors [60]. Further research is needed to evaluate the potential of this stem cell lineage in liver regenerative setups.

*4.2.4. Liver stem/progenitor cells (LSPCs)*

Hepatoblasts being bipotent are capable of self-renewal and differentiation into cholangiocytes and hepatocytes. In contrast to ESCs and MSCs, both of which need to go through sequential differentiation to develop into mature hepatocytes, LSPCs have a destined fate. Hence, they carry significant potential to be used in liver regenerative medicine. LSPCs can undergo several rounds of proliferation. These cells have the potential to differentiate into hepatic and biliary cell lineages and to repair the damaged liver tissue [50, 61]. LSPCs are thought to be the cells that do not contribute to the routine liver yields. Instead, they appear in advance stages of liver injury such as primary biliary cirrhosis and nonalcoholic cirrhosis [21]. Many properties of embryonic hepatoblasts are shared by LSPCs. Certain surface markers help in selective isolation of LSPCs via immune selection. They express epithelial cell adhesion molecules (EpCAM) and have been isolated against this surface marker [11] from fetal as well as adult human liver [62]. Differentiation of EpCAM-positive cells can yield both hepatocytes and cholangiocytes [63, 64]. Clinical trials of EpCAM-positive LSPCs are given in **Table 1**.

LSPCs, on the other hand, have certain limitations which hinder their application in liver regenerative medicine. First of all, these cells are present in a very small quantity in the adult human liver making it unproductive to isolate them on the basis of their markers. Our research group had addressed this problem in a recently published study, where BMSCs were differentiated toward oval cell-like cells. These oval cell-like cells were comparable to control oval cells in their efficiency to reduce liver injury [65]. Another major issue associated with LSPCs is their great potential to induce hepatic tumorigenicity. Presently, this is a major limiting factor for

their utilization in liver therapeutics and regenerative medicine. Notably, human liver progenitor cells have been found to be present and contributing in the development of nonalcoholic steatohepatitis in pediatric and adult human patients. They are supposed to be playing fibrogenic role in such cases as reported by Sobaniec-Łotowska et al. [66]. Comprehensive research at preclinical level is required to probe into these issues properly to understand the appropriateness of these cells for clinical trials.

*4.2.5. Induced pluripotent stem cells (iPSCs)*

The establishment of iPSCs by reprogramming somatic cells through certain transcription factors (Oct-3/4, Sox2, Nanog, c-Myc, Klf-4) has proven a potential new source of stem cells. These cells exhibit properties essential for ESCs and have the potential to differentiate into the derivatives of all three germ layers [67]. However, iPSCs avoid the ethical issues related to ESCs since no human embryo is used for their production [3]. iPSCs being autologous in nature also evade the problem of immune rejection. Although there are unlimited sources for iPSCs generation, to ensure a relatively homogeneous hepatocyte culture, the use of hepatocytes or/and other endodermal cells is recommended. It can play an important role as cells carry an "epigenetic memory" allowing the iPSCs to differentiate toward cells of definitive germ layer [68].

Permanent retroviral integration, a process which was initially used by Takashi and coworkers in 2007 [69] is one of the earliest methods used for iPSCs production. With advancement in the field, it is possible to generate iPSCs without using retroviral transfection. Nowadays, a number of methods such as excisable viral transfection [70], microRNA transfection [71], episomal plasmid transfection [72], and mRNA transfection [73] are being harnessed for the production of functionally efficient iPSCs. Once generated, iPSCs can be directed to differentiate toward definitive endoderm which will differentiate into hepatoblasts and finally into hepatocytes in a sequential manner involving various growth factors, cytokines, and signaling pathways as described previously in this chapter. The resultant hepatocyte-like cells are more like fetal hepatocytes rather than mature hepatocytes, a phenomenon shared by all the stem cell-generated hepatocytes [3]. Although an efficient source of autologous transplantation, iPSCs-derived hepatocytes have certain shortcomings as well.

## 5. Tissue engineering and liver cirrhosis

Cell-based therapies have shown promising results in the improvement of liver cirrhosis. However, inefficient engraftment of cells due to surrounding conditions of diseased liver results in variable outcomes [3]. Tissue engineering, a recent advancement in liver regenerative medicine, is dedicated in deriving the ways to escape the problems associated with direct cell-based therapies. It mainly focuses on the development of biocompatible scaffolds and extracorporeal liver devices suitable for either in vitro or in vivo applications. Schematic representation of key approaches used for liver tissue engineering is shown in **Figure 3** and discussed in detail with their merits and relevant complications in the following section.

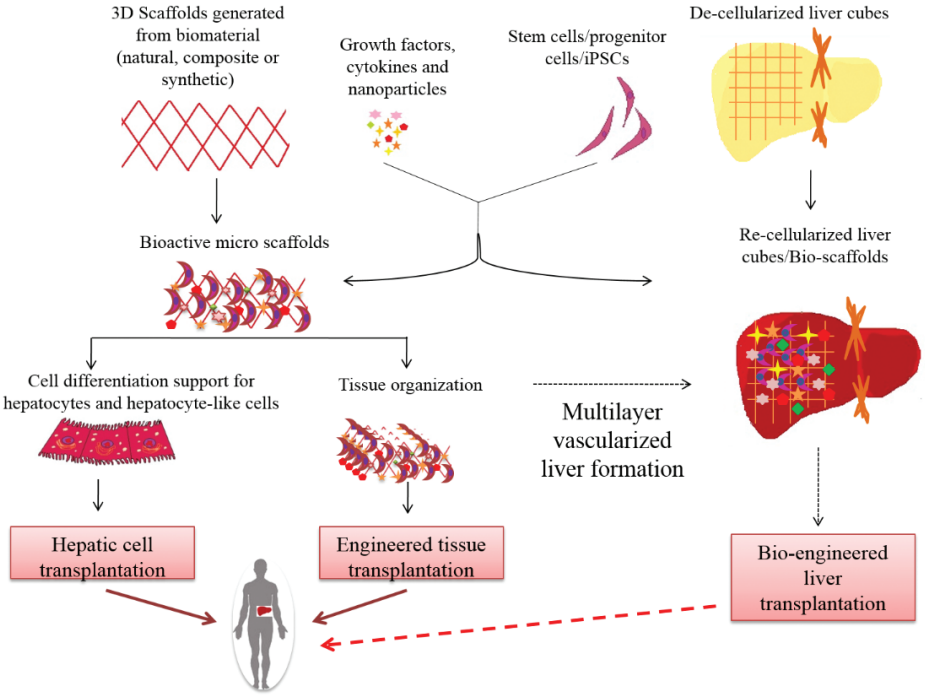


**Figure 3.** Schematic diagram of liver tissue engineering. Solid lines show the approaches already ongoing whereas dotted lines indicate the proposed mechanisms.

### 5.1. Generation of bioactive scaffolds

Bioactive scaffolds are those that have the ability to elicit cell growth and differentiation. In modern tissue engineering, bioactive scaffolds are so much advantageous as they mimic the natural ECM environment of the liver. One of the major components of these scaffolds is a structural protein collagen normally found in skin, bone, and cartilage [90]. Collagen highly supports attachment, proliferation, differentiation, growth, and migration of cells. Further, collagen-based bioscaffolds have shown in vitro differentiation of embryoid bodies derived from embryonic stem cell into hepatocyte-like cells [91, 92]. Hyaluronic acid is another important component of the extracellular matrix. It is involved in the regulation of cell proliferation and expansion. The immature and mature hepatocytes of fetal and adult liver cells express surface receptors for hyaluronic acid, that is CD44 [93]. By utilizing this property of hepatocytes, hydrogels consisting of hyaluronic acid and its derivatives are synthesized possessing more adhesive power for hepatocytes. They can retain viability of hepatocytes for 4 weeks [93].

Other natural biomaterials being utilized in the formation of bioactive scaffolds are alginate, chitin, chitosan, silk, matrigel, and sponge. Its best example is silk-fibroin-based microfluidic devices that successfully supported the growth and differentiation of HepG2 cells [94]. Hepatic

organoids and smaller parts of tissues can be grown from porcine hepatocytes on the matrix, consisting of albumin and chitosan (a deacetylated form of chitin) [95]. Scaffold containing chitosan nanofibers associated with the glucose residues showed prolonged metabolic activity of cluster of cells originated from hepatocytes [96]. Hydrogels formed by the natural biomaterials such as alginate and matrigels are more biocompatible and improve the seeding potency of hepatocytes. The basal membranes of murine chondrosarcoma are used for extraction of proteins (laminin, heparan sulfate proteoglycan, collagen type IV) that are used in the formation of matrigels. Hepatocytes initially started to grow in scaffolds containing matrigels into shapeless clusters of cells followed by their implantation in natural organ [97].

However, it has not yet been recognized that which composition would provide the best physicochemical characteristics for defined growth pattern of hepatocytes. Moreover, due to xenogeneic and tumorigenic origin of matrigels, they are not considered good for tissue engineering of liver. Although utilization of natural polymers in three-dimensional (3D) scaffolds creates some histoarchitectural features that help a lot in the generation of cell-to-cell and cell-to-matrix interactions, uncontrollable physicochemical properties, degradability, lack of regenerative ability, and inconsistent mechanical properties halt its clinical implication.

### 5.2. Synthetic polymers used in liver tissue engineering

In comparison to natural biomaterials used in tissue engineering, synthetic materials provide a wide range of properties and a better control over them. Their biocompatibility and biodegradability can be tuned easily. Scaffolds containing biodegradable polymers facilitate regeneration, transplantation, and degradation of cells on time. Commonly used biodegradable polymers are polylactic acid, polyglycolic acid, polyanhydrides, polyfumarates, polyorthoesters, polycaprolactones, poly- L–lactic acid, and polycarbonates [98].

A synthetic chemical polyglycolate–polylactate used in 3D scaffolds can turn fetal hepatoblasts to mature hepatocytes [99–101]. The main limitations of polyglycolate–polylactate are chemical unpredictability, surface corrosion, and hydrophobicity [102]. However, chemical instability of poly (alpha-hydroxy) acids results in the formation of hydrolysis products, which can induce inflammatory responses. The chemical modification of polymers (e.g. the incorporation of proteins and special bioactive domains) increases the biocompatibility of bioengineered matrices and improves scaffold adhesion properties stimulating cell attachment and migration, thereby, facilitating liver tissue repair [103]. 3D hepatocyte cultures can also be grown successfully in polyurethanes. Polyurethane foams are used to grow hepatocytes and hepatocyte-like cells in bioreactors. Highly functional multicellular structures are formed within the pores of these polyurethane foams [104]. Because of these characteristic polyurethane foams are widely used in 3D scaffolds for the production of bioartificial liver [105].

### 5.3. Implementation of nanotechnology and microchip devices in tissue engineering

Nanotechnology and microchip devices have tremendous use in liver tissue engineering. Microfluidic devices containing very small volumes of cells, effector molecules, ECM, and so on are used to produce natural biochemical environment around the cells so that they may behave as they do in natural organ [106]. Using the microbioreactors, microcapsule fabrication is done

that leads to the encapsulation of hepatic cells and their precursors. In these special kinds of bioreactors, the regular supply of oxygen, water, and nutrients is ensured and metabolic wastes are eliminated. These capsules are made of polydimethylsiloxane and its derivatives because they are highly permeable to water. The polydimethylsiloxane capsules and microspheres of alginate have showed efficient growth of encapsulated hepatocytes that were seeded on them due to its radical perfusion properties. Due to its remarkable properties, polydimethylsiloxane is a promising tool for bioartificial liver system [72].

To estimate cytotoxic effects of drugs on liver cells, 3D microfluidic cell panels have also been introduced. These panels create the natural environment for cells as they are made up of porous hydrogels and are lined with hepatocytes. These pores are taken as capillaries by the cells. Various pharmacokinetic models are being studied with the help of these panels [107, 108].

Speaking collectively, complex microarchitecture of liver tissues having proper cell to cell interactions and supply of cells with oxygen and nutrients are produced from biologically produced microorgans of liver. These microorgans are produced ultimately from bioactive microscaffolds; 3D hepatocyte panels [109].

### 5.4. Organ-based regeneration of liver

The development of whole organ using different techniques in tissue engineering is remarkable and this decreases the problems related to shortage of donor organs for transplant and immunosuppression. In order to build a functional liver organ, the first and foremost needed is a scaffold. Among many of the trialed materials for scaffolds, porcine/murine-based scaffolds have proved better. Second, what is needed is the presence of extracellular matrix in the scaffolds to provide the hepatocytes with their niche for their optimal growth and regulation of cellular behaviors [110, 111].

Complete decellularization of native organ is achieved via detergent perfusion for 24–48 hours, in order to get a xenogeneic scaffold. A point that must be mentioned while decellularization is: ECM should not be damaged and it should have under 50 ng double-stranded DNA/mg of ECM to avoid immune rejection [112]. After decellularization, recellularization of xenogenic scaffold with highly functional hepatocytes is done. These cells are obtained either from deceased donor grafts or from partial hepatectomy. However, it is difficult to obtain an appropriate volume of cells. The adult hepatocytes are not considered good for organ regeneration because they show poor in vitro proliferation. Fetal liver cells show high in vitro rate of proliferation but they are not easy to obtain. The human-derived cell lines that show exponential growth in vitro also cannot be used for implantable organs as they pose the threat of metastasis [113, 114]. Porcine hepatocytes remained successful in BAL system but due to immunogenic rejection they cannot be used for organ bioengineering [5]. Human-derived autologous stem cells, that is iPSCs, are capable of producing liver-specific proteins but they produce the albumin at a lower rate than in adult human liver so they are also not a good choice. However, human bone marrow cells are showing promising results in vitro, though they are not yet tested clinically [115].

The recellularization of scaffolds fitted in the tissue cultures of organ chambers is done either by direct parenchymal injections or by single or multistep perfusion in physiological

pressure. As a proof of whole liver decellularization and recellularization concept a rat model was utilized for the proliferation of adult rat hepatocytes. Proliferation was confirmed by different markers. Ninety percent of hepatectomized rat models that were given spheroid tissue-engineered liver showed an increased survival period from 16 to 72 hours. But to their dismay, the rats died of the small-for-size syndrome [116, 117].

Besides facing problem in the selection of most suitable cell lines, another hurdle is to develop a vascular network for the support of cell aggregates [118]. Organ bioengineering offers a hopeful way to get out of complications associated with liver cirrhosis. The best scaffold onto which organ is tissue engineered is a decellularized xenogenic scaffold having intact network of ECM. Studies are being focused on the determination of ideal cell types for humans. Deep research is also going on to find the optimal cell seeding techniques and cell volume required to sustain necessary functions [5].

## 6. Conclusion

In conclusion, the field of regenerative medicine has taken a successful initiative toward the ultimate solution of end-stage liver diseases. Particularly, the dynamism of various cell-based therapies has arisen much hope and facilitated the development of more challenging tissue engineering. Initially, tissue engineering focused on the use of natural and synthetic scaffolds to grow hepatocytes and develop liver tissues. Currently, much work is ongoing to create liver microorgans to organoids. Crucial aim of future research is to construct whole bioengineered liver. In this regard, the use of decellularized livers has been proposed to create liver organoids leading to the construction of whole bioengineered liver. However, organ bioengineering faces the problems of selection of suitable cell type and appropriate development of a vascular network, which will support cell aggregates. Major challenges associated are the determination of suitable cell type, optimal cell volume, and seeding techniques required to endure essential hepatic functions. The current scenario propels to conduct much more experimental work to successfully construct whole bioengineered liver and its effective clinical applications to replace liver transplantation.

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Chapter 13

# The Promising Role of Anti-Fibrotic Agent Halofuginone in Liver Fibrosis/Cirrhosis

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Additional information is available at the end of the chapter

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**Abstract**

Liver fibrosis is a complex inflammatory and fibrogenic process that results from chronic liver injury and represents an early step in the progression of cirrhosis. Several cell types [hepatic stellate cells (HSCs), hepatocytes, liver sinusoidal endothelial cells (LSECs), and Kupffer cells (KCs)], cytokines [platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, interferons (IFNs), interleukins (ILs)], oxidative stress, and microRNAs (miRNAs) are involved in the initiation and progression of liver fibrosis/cirrhosis. Generally, liver fibrosis begins with the stimulation of inflammatory immune cells to secrete cytokines, growth factors, and other activator molecules. These chemical mediators direct HSCs to activate and synthesize large amounts of extracellular matrix (ECM) components. Therefore, HSC activation is a pivotal event in the development of fibrosis and a major contributor to collagen (specifically type I) accumulation. The inhibitory effect of halofuginone on collagen type α1(I) synthesis and ECM deposition has been shown in several experimental models of fibrotic diseases. Halofuginone inhibits TGF-β–induced phosphorylation of Smad3, which is a key phenomenon in the fibrogenesis. It also regulates cell growth and differentiation, apoptosis, cell migration, and immune cell function in liver fibrosis/cirrhosis. This review discusses the etiology and mechanisms of liver fibrosis/cirrhosis and the promising role of anti-fibrotic agent halofuginone.

**Keywords:** liver fibrosis, liver cirrhosis, hepatic stellate cells, pathogenesis, anti-fibrotic, halofuginone

## 1. Introduction

Liver cirrhosis is the end-stage condition of several chronic liver diseases, and fibrosis is the critical pre-stage of cirrhosis. On a worldwide perspective, liver cirrhosis can be induced by

a number of well-defined etiological causes/factors or conditions such as chronic infection by hepatitis B, C viruses, chronic alcoholism and/or chronic exposure to toxins or drugs, infections, chronic exposure to altered metabolic conditions, inherited metabolic diseases such as hematochromatosis and Wilson's disease, auto-immune diseases such as primary biliary cirrhosis, and auto-immune hepatitis [1–3]. These etiologies may work separately or in combination with each other to produce cumulative effects. While the causes of liver cirrhosis are multifactorial, there are some pathological characteristics that are common to all cases of cirrhosis, including degeneration and necrosis of hepatocytes, replacement of healthy liver parenchyma by fibrotic scar tissues and regenerative nodules, and loss of liver function [4–7].

Fibrosis is characterized by high levels of extracellular matrix (ECM, non-functional connective tissue) components extremely rich in collagen type I. The matrix metalloproteinases (MMPs, matrix degradation enzymes), and the tissue inhibitor of metalloproteinases (TIMPs) play a crucial role in the fine regulation of ECM turnover, which is altered in most pathological states associated with liver fibrosis [8]. The key cellular mediator of fibrosis comprises the activated hepatic stellate cells (HSCs), which serve as the primary ECM-producing cells. HSCs, which play a key role in the development of liver fibrosis [9, 10], are activated by several inflammatory cytokines and growth factors in a paracrine and autocrine manner [11, 12].

Liver fibrosis and cirrhosis are dynamic and highly integrated molecular, tissue and cellular processes that can progress and regress over time [13] and that require cellular cross-talk between various liver cell types [14]. At early stages of fibrosis, initiating signals [such as DNA, reactive oxygen species (ROS)], responding cells [Kupffer cells (KCs), platelets, liver sinusoidal endothelial cells (LSECs)], and soluble mediators [such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β] induce accompanying wound-healing responses to liver injury. With time, cells, cytokine responses, and ECM components become more specialized but continue to have strong interactions with each other [15].

Halofuginone is a non-toxic plant alkaloid [7-bromo-6-chloro-3-(3-hydroxy-2-piperidine)-2-oxopropyl-4(3H)-quinazoline] isolated from the roots of *Dichroa febrifuga*, and is used worldwide as an anti-parasitic drug [16]. Independent of this effect, halofuginone was found to be a potent inhibitor of collagen type $\alpha$1 (I) gene expression [17], which was demonstrated in a broad range of cell types both *in vitro* and *in vivo* [16–20]. Due to its inhibitory effects on collagen synthesis (collagen type $\alpha$1) and ECM deposition, halofuginone treatment was used in several experimental disease models characterized by excessive collagen accumulation, such as pulmonary, pancreatic and renal fibrosis [21–23], scleroderma and chronic graft-versus-host disease [24], post-operative peritendinous and abdominal adhesions [25, 26], urethral and esophageal strictures [27, 28], wound repair [29], burn injury [30], renal injury [31, 32], injury-induced arterial intimal hyperplasia [33], colitis [34], and liver fibrosis and cirrhosis [35–39]. Although the exact anti-fibrotic mechanism of halofuginone is not well understood, it was found that halofuginone affects collagen synthesis probably by inhibiting TGF-β-mediated Smad3 (intracellular protein) activation [40]. Halofuginone also regulates cell growth and differentiation, apoptosis, cell migration, and immune cell function [41]. It prevents concanavalin A-induced liver fibrosis by affecting T helper 17 (Th17) cell differentiation, which suggests a direct connection between the myofibroblasts/fibrosis pathway and

the Th17 pro-inflammatory pathway [38]. In addition, halofuginone treatment effectively inhibits the delayed-type hypersensitivity response, indicating suppression of T cell–mediated inflammation *in vivo* [42]. Moreover, it is a potent inhibitor of nuclear factor (NF)-κB, pro-inflammatory cytokines and p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation in activated T cells *in vitro* [42]. Also, it inhibits HSC proliferation and migration and up-regulates their expressions of fibrolytic MMP-3 and -13 via activation of p38 MAPK and NF-κB [43].

Although there are no highly effective anti-fibrogenic agents currently available, the potential candidates that can specifically inhibit ECM components in general and specifically inhibit collagen type I in particular, are considered to be promising for the prevention and treatment of liver fibrosis/cirrhosis. The present review aims to clarify the etiology and mechanisms of liver fibrosis/cirrhosis and focus on the anti-fibrotic potential of a novel and promising agent, halofuginone.

## 2. Role of different cell types in liver fibrosis/cirrhosis

The liver is composed of parenchymal cells (hepatocytes) and non-parenchymal cells (HSCs, LSECs, and KCs). Both parenchymal and non-parenchymal cells are involved in the initiation and progression of liver fibrosis/cirrhosis (**Table 1**).

| Cell types | Role in liver fibrosis/cirrhosis | References |
|---|---|---|
| Hepatic stellate cells (HSCs) | Main function is storage of vitamin A and other retinoids | [7, 44] |
| | Undergo a phenotypic switch from a quiescent type into an activated type (myofibroblast-like cells) by several inflammatory cytokines | [46] |
| | Activated HSCs are major contributors to collagen accumulation | [47, 48] |
| Hepatocytes | Hepatocyte-derived apoptotic bodies stimulate secretion of fibrogenic cytokines from KCs and promote HSC activation | [50–53] |
| | Hypoxic hepatocytes become a primary source of TGF-β in cirrhotic stage | [55] |
| Liver sinusoidal endothelial cells (LSECs) | Defenestration and capillarization of LSECs lead to impaired substrate exchange and HSC activation | [57, 61, 62] |
| | Secrete IL-33 to activate HSCs | [63] |
| Kupffer cells (KCs) | Activated KCs secrete inflammatory cytokines, promote HSC activation, and stimulate cell proliferation | [65–69] |
| | KC-derived TGF-β1 stimulates proliferation and collagen formation of HSCs | [66] |
| | Activated KCs kill HSCs by a caspase 9-dependent mechanism via TRAIL | [72, 73] |

*Abbreviations*: TGF-β, transforming growth factor-β; IL, interleukin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

**Table 1.** Role of different cell types in liver fibrosis/cirrhosis.

### 2.1. Hepatic stellate cells (HSCs)

HSCs are one of the non-parenchymal cells of the liver located in the perisinusoidal space (space of Disse) between hepatocytes and sinusoidal endothelial cells. HSCs are also known as fat-storing cells, perisinusoidal cells, lipocytes, or vitamin A-rich cells, and their main function is storage of vitamin A and other retinoids [7, 44]. HSCs show two different phenotypes: quiescent type in the healthy liver and activated type in the diseased one. Quiescent HSCs mostly function as vitamin A reserves [45]. However, in response to liver injury, inflammatory cytokines such as tumor necrosis factor (TNF)-$\alpha$, TGF-$\beta$, interleukin (IL)-1, and PDGF promote HSCs to undergo a phenotypic switch from a quiescent, vitamin A storing cell into proliferative, $\alpha$-smooth muscle actin ($\alpha$-SMA)-positive, myofibroblast-like cells which contribute to fibrosis by producing the abnormal ECM components [46]. Therefore, HSC activation is a pivotal phenomenon in initiation and progression of liver fibrosis and a major contributor to collagen accumulation [47, 48].

### 2.2. Hepatocytes

Hepatocytes are the primary parenchymal component of the liver and play an important role in fibrosis/cirrhosis. They are the main targets of several hepatotoxic agents including hepatitis viruses, alcohol metabolites, and bile acids [11]. Liver injury either promotes apoptosis or triggers compensatory regeneration of hepatocytes [49]. Hepatocyte-derived apoptotic bodies stimulate secretion of fibrogenic cytokines from KCs and promote HSC activation via interaction of toll-like receptor (TLR)-9 with DNA, which is released from apoptotic hepatocytes [50–53]. On the other hand, activated HSCs also act as phagocytes and phagocytize hepatocyte apoptotic bodies, which promote myofibroblasts survival and fibrogenesis [54]. Therefore, apoptosis of hepatocytes is a crucial event in liver injury and contributes to tissue inflammation, fibrogenesis, and development of cirrhosis. Also, in the cirrhotic stage, hypoxic hepatocytes become a primary source of TGF-$\beta$, which may augment liver fibrosis [55].

### 2.3. Liver sinusoidal endothelial cells (LSECs)

LSECs constitute the sinusoidal wall, also known as endothelium, or endothelial lining. The main characteristic of LSECs is having the fenestrae on the surface of the endothelium [56, 57]. The endothelial fenestrae control exchange of fluids, solutes, and particles between sinusoidal blood and hepatocytes [58]. In the healthy liver, the fenestrated endothelial cells prevent HSC activation through vascular endothelial growth factor-stimulated nitric oxide production [59]. However, LSECs have high endocytotic capacity [56, 60]. Upon liver injury, defenestration and capillarization of LSECs lead to impaired substrate exchange which is the major cause of hepatic dysfunction [57, 58] and HSC activation [61, 62]. It has been also revealed that LSECs can secrete the cytokine IL-33 to activate HSCs and promote liver fibrosis [63].

### 2.4. Kupffer cells (KCs)

KCs, also called stellate macrophages, are interspersed throughout the liver, situated within the sinusoids. KCs are responsible for the removal of circulating microorganisms, immune

complexes, and debris from the blood stream. They are usually activated by many injurious factors such as viral infection and alcohol [64]. Activation of KCs is a key phenomenon in initiation and preservation of liver fibrosis. Activated KCs express chemokine receptors, secret inflammatory cytokines (such as TNF-$\alpha$, IL-1, IL-6) and serve as antigen-presenting cells, which lead to progression of fibrosis [65–68]. KCs are also involved in the activation of HSCs and formation of liver fibrosis. For example, KC-conditioned medium promotes activation of cultured rat HSCs with enhanced ECM production and stimulates cell proliferation via induction of PDGF receptors on the membrane of HSCs [69]. KC-derived TGF-$\beta$1 stimulates proliferation and collagen formation of HSCs in a rat model of alcoholic liver fibrogenesis [66]. Moreover, macrophage ablation has been shown to attenuate liver fibrosis. For example, gadolinium chloride-mediated depletion of KCs has been shown to result in attenuation of carbon tetrachloride ($CCl_4$)-induced fibrosis in rats with prevention of the increased TGF-$\beta$ expression [70]. Conversely, KCs produce interstitial collagenase MMP-13 when treated with gadolinium chloride, which reduces ECM deposition during experimental liver fibrosis [71]. In addition, activated KCs can effectively kill HSCs by a caspase 9-dependent mechanism via possible involvement of TNF-related apoptosis-inducing ligand (TRAIL) [72, 73].

## 3. Role of cytokines in liver fibrosis/cirrhosis

Cytokines, which mediate several immune and inflammatory reactions, are small signaling proteins that facilitate intercellular communication between various cells. They function through cell-surface receptors, and down-stream signaling induces an alteration of cell functions. Liver fibrosis/cirrhosis is a result of interaction of a complex network of cytokines, which modify activities of circulating immune cells, HSCs, KCs, LSECs, and hepatocytes. The role of cytokines in liver fibrosis/cirrhosis is summarized in **Table 2**.

### 3.1. Platelet-derived growth factor (PDGF)

PDGF is one of the most potent mitogen for HSCs isolated from mouse, rat, or human liver [74]. PDGF and its receptors are significantly overexpressed in fibrotic tissues, and its activity increases with the degree of liver fibrosis [75, 76]. Hepatocyte damage resulting from factors, such as viruses, chemicals, or hepatotoxins, can induce KCs to synthesize and release PDGF [77]. When PDGF binds to its specific receptor on the membrane of HSCs, it activates corresponding signal molecules and transcription factors, leading to the activation of its down-stream target genes and activation of HSCs [74]. PDGF has been shown to up-regulate the expression of MMP-2, MMP-9, and TIMP-1, and inhibit collagenase activity, thereby decreasing ECM degradation [78].

### 3.2. Transforming growth factor (TGF)-β

Among fibrotic mediators, TGF-$\beta$ is one of the most important pro-fibrotic cytokine. The direct targets in TGF-$\beta$ pathway, Smads (especially Smad3) are critical mediators in fibrogenesis [79, 80]. The intracellular effectors of TGF-$\beta$ signaling, the Smad proteins, are activated by receptors and

| Mediators | Mechanism of action | References |
|---|---|---|
| Platelet-derived growth factor (PDGF) | Activates HSCs | [74] |
| | Up-regulates expression of MMP-2, MMP-9, and TIMP-1 and inhibits collagenase activity | [78] |
| Transforming growth factor (TGF)-β | Stimulates HSC activation | [81, 82] |
| | Induces expression of matrix-producing genes, inhibits ECM degradation, and promotes TIMPs | [84, 85] |
| | Inhibits DNA synthesis and induces apoptosis of hepatocytes | [86–88] |
| Tumor necrosis factor (TNF)-α | Induces hepatocyte death by apoptosis | [90] |
| | Activates HSCs and stimulates ECM synthesis | [91, 92] |
| | Induces/reduces apoptosis of activated HSCs | [73, 93] |
| | Reduces glutathione and inhibits pro-collagen α1 mRNA expression | [94] |
| Interferons (IFNs) | | |
| IFN-α | Triggers apoptosis of HSCs | [96] |
| | Elicits an anti-apoptotic effect on activated HSCs | [100] |
| IFN-β | Decreases α-SMA and collagen expression and inhibits HSC activation through inhibition of TGF-β and PDGF | [97] |
| IFN-γ | Reduces ECM deposition by inhibiting HSC activation | [98] |
| | Exerts a pro-apoptotic effect on activated HSCs | [100] |
| Interleukins (ILs) | | |
| IL-1 | Activates HSCs and stimulates them to produce MMP-9, MMP-13 and TIMP-1 | [102] |
| | Increases MCP-1 in hepatocytes and augments TLR-4-dependent up-regulation of inflammatory signaling in macrophages | [105] |
| IL-17 | Regulates production of TGF-β1 by KCs, induces activation of HSCs and induces production of collagen and α-SMA in HSCs via STAT3 pathway | [108] |
| IL-6 | Attenuates hepatocyte apoptosis and induces regeneration of hepatocytes through NF-κB pathway | [112] |
| IL-10 | Inhibits expression of TGF-β1, MMP-2 and TIMP-1 | [115] |
| | Inhibits HSC activity | [117] |
| | Reduces TGF-β1, TNF-α, collagen α1, and TIMP mRNA up-regulation | [120] |
| IL-22 | Inhibits hepatocyte apoptosis via STAT3 | [121, 122] |
| | Induces HSC senescence | [123] |

*Abbreviations*: HSC, hepatic stellate cell; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ECM, extracellular matrix; SMA, smooth muscle actin; MCP, monocyte chemoattractant protein; TLR, toll-like receptor; KC, Kupffer cell; STAT, signal transducer and activator of transcription; NF-κB, nuclear factor-κB.

**Table 2.** Role of cytokines in liver fibrosis/cirrhosis.

translocate into the nucleus, where they regulate transcription [79]. The main effect of TGF-β is to stimulate HSC activation, and the TGF-β autocrine cycle in activated HSCs is an important positive feedback to the progression of liver fibrosis [81, 82]. Though the main source of TGF-β in fibrotic liver is activated HSCs, LSECs, KCs, and hepatocytes also contribute to synthesis of this growth factor [83]. The level of TGF-β1 expression is increased during liver fibrosis and reaches a maximum at cirrhosis [55]. TGF-β1 induces expression of the matrix-producing genes, inhibits ECM degradation, and promotes TIMPs, leading to excessive collagen accumulation and promoting the development of liver fibrosis [84, 85]. Furthermore, TGF-β1 has been shown to inhibit DNA synthesis and induces apoptosis of hepatocytes. In particular, TGF-β1-induced apoptosis is thought to be responsible for tissue loss and decrease in liver size seen in cirrhosis [86–88].

### 3.3. Tumor necrosis factor (TNF)-α

TNF-α is a pro-inflammatory cytokine produced by different cell types. However, it is mainly produced by activated KCs in the liver. TNF-α is an important mediator in several processes such as cell proliferation, inflammation, and apoptosis [89]. TNF-α can induce cell death by apoptosis, and KCs can be stimulated by apoptotic hepatocytes to produce more TNF-α [90]. Furthermore, TNF-α plays an essential role in the HSC activation and ECM synthesis in liver fibrosis [91, 92]. TNF-α may act as surviving factor for activated rat HSCs by up-regulating the anti-apoptotic factors (NF-κB, bcl-xL, and p21WAF1) and by down-regulating the pro-apoptotic factor (p53) [93]. On the other hand, TNF-α can induce apoptosis in HSCs [73]. It has been also demonstrated that TNF-α shows anti-fibrogenic effect in rat HSCs by reducing glutathione and inhibiting pro-collagen α1 mRNA expression [94].

### 3.4. Interferons (IFNs)

IFNs are potent pleiotropic cytokines that broadly alter cellular functions in response to viral and other infections. Leukocytes synthesize IFN-α and IFN-β in response to viruses, and T cells secrete IFN-γ upon stimulation with various antigens and mitogens. Although the primary action of IFN-α is to eradicate viruses, patients with hepatitis C treated with IFN-α exhibit a regression of liver fibrosis even if viral eradication is not achieved [95], indicating that IFN-α itself has anti-fibrotic activity via triggering the apoptosis of HSCs [96]. IFN-β treatment decreases α-SMA and collagen expression and inhibits HSC activation through inhibition of TGF-β and PDGF pathways [97]. Similarly, IFN-γ reduces ECM deposition *in vivo* by inhibiting HSC activation [98] via TGFβ1/Smad3 signaling pathways [99]. Interestingly, IFN-α and IFN-γ may exert opposite effects on apoptosis in HSCs. IFN-α was shown to elicit an anti-apoptotic effect on activated HSCs, whereas IFN-γ was found to exert pro-apoptotic effect on HSCs by down-regulating heat-shock protein 70 [100].

### 3.5. Interleukins (ILs)

ILs are immunomodulatory cytokines that are critically involved in the regulation of immune responses. They are produced by a variety of cell types such as $CD4^+$ T lymphocytes, monocytes, macrophages, and endothelial cells. KCs and LSECs can rapidly produce ILs in response to liver injury. ILs can have pro- and anti-inflammatory functions in chronic liver diseases, dependent on the inflammatory stimulus and, the producing and the responding cell type.

The main function of pro-inflammatory ILs is to stimulate immune responses that result in the elimination of invading pathogens or damaged cells. On the other hand, anti-inflammatory ILs are produced to protect the host's body from exaggerated immune responses and to limit organ damage. As soon as the pathogenic stimuli are removed, ILs production is no longer needed, and inflammation diminishes. If the stimulus continues, inflammation can become chronic and induce a variety of inflammatory diseases [101].

IL-1 is a pro-inflammatory and pro-fibrotic cytokine that directly activates HSCs and stimulates them to produce MMP-9, MMP-13, and TIMP-1, resulting in liver fibrogenesis [102]. IL-1 receptor-deficient mice exhibits ameliorated liver damage and reduced fibrogenesis [102]. Similarly, IL-1 receptor antagonist protects rats from developing fibrosis in dimethylnitrosamine-induced liver fibrosis [103]. Lack of IL-1$\alpha$ or IL-1$\beta$ also makes the mice less susceptible to develop liver fibrosis in experimental model of steatohepatitis [104]. It has been also shown that IL-1$\beta$ at physiological doses increases the inflammatory and prosteatotic chemokine monocyte chemoattractant protein (MCP)-1 in hepatocytes, and augments TLR-4-dependent up-regulation of inflammatory signaling in macrophages [105]. Thus, IL-1 is an important participant, along with other cytokines, and controls the progression from liver injury to fibrogenesis.

Another pro-inflammatory and pro-fibrotic cytokine IL-17 has been reported to be involved in many immune processes, most notably in inducing and mediating pro-inflammatory responses. Its expression increases with increasing degree of liver fibrosis [106, 107], suggesting that IL-17 may not only induce inflammation but also contribute to disease progression and chronicity [106]. IL-17 regulates production of TGF-$\beta$1 by KCs, which in turn, induces activation of HSCs into myofibroblasts, and further facilitates differentiation of IL-17 expressing cells [108]. Also, IL-17 directly induces production of collagen and $\alpha$-SMA in HSCs via the signal transducer and activator of transcription (STAT)3 signaling pathway [108]. Furthermore, abrogation of IL-17 signaling by deletion of IL-17RA protects mice from fibrogenesis [108]. Similarly, blockade of endogenous IL-17 with neutralizing IL-17-specific antibody reduces liver fibrosis, whereas treatment with recombinant IL-17 increases fibrosis development [109].

IL-6 is a pleiotropic cytokine, which may affect differentiation of fibroblast to myofibroblast, and it plays an important role in fibrotic diseases [110, 111]. On the other hand, IL-6 has beneficial effects for the liver. For example, IL-6 reduces $CCl_4$-induced acute and chronic liver injury and fibrosis [112]. Also, it attenuates hepatocyte apoptosis and induces regeneration of hepatocytes through NF-κB signaling pathway [112]. In an experimental model of concavaline A-induced hepatitis, IL-6 pretreatment protects mice from liver injury. This protection requires gp130 signaling in hepatocytes and is mediated via the gp130/STAT3 signaling cascade [113]. Furthermore, systemic injection of IL-6 followed by intrahepatic transplantation of mesenchymal stem cells is also able to reduce hepatocyte apoptosis and liver fibrogenesis after $CCl_4$ treatment [114].

IL-10 is one of the major anti-inflammatory cytokines, with tissue protective functions during fibrogenesis. It down-regulates the pro-inflammatory response and has a modulatory effect on liver fibrogenesis [115, 116]. IL-10 has been shown to exert anti-fibrotic effects through inhibiting HSC activity [117]. IL-10-deficient mice show higher liver fibrosis with larger

inflammatory infiltrates in $CCl_4$-induced liver fibrosis compared to wild-type mice [118, 119]. IL-10 gene therapy reverses $CCl_4$-induced murine liver fibrosis by inhibiting the expression of TGF-β1, MMP-2, and TIMP-1 [115]. Additionally, IL-10 gene therapy reverses liver fibrosis and prevents cell apoptosis in a thioacetamide-treated murine liver, and reduces TGF-β1, TNF-α, collagen α1, and TIMP mRNA up-regulation, suggesting a therapeutic potential for treatment with IL-10 [120].

IL-22 is known to play important roles in the modulation of tissue immune responses to inflammation. It reduces inflammation-induced damage of hepatocytes both *in vitro* and *in vivo* by promoting their survival and inhibiting apoptosis [121]. This protective function is dependent on STAT3 signaling, as STAT3-deficient mice were not protected when treated with IL-22 [122]. Similarly, in $CCl_4$-induced liver fibrogenesis, IL-22 is protective through induction of senescence in HSCs via STAT3 signaling pathway [123]. Moreover, IL-22 is also involved in the restoration of functional liver mass after organ damage. Liver progenitor cells have been shown to express IL-22R, and IL-22 derived from inflammatory cells induces proliferation of liver progenitor cells [124].

## 4. Role of oxidative stress in liver fibrogenesis

Oxidative stress is caused by an imbalance between production of ROS and their elimination by anti-oxidant defenses [125]. As liver is an essential organ for detoxification and nutrients metabolism, it is more vulnerable to oxidative stress [125]. Oxidative stress-related molecules and pathways modulate tissue and cellular events involved in the liver fibrogenesis [126]. The generation of ROS plays a crucial role in producing liver damage and initiating liver fibrogenesis [126]. Oxidative stress disrupts lipids, proteins and DNA, induces necrosis and apoptosis of hepatocytes, resulting in the initiation of fibrosis [127]. ROS stimulate the production of pro-fibrogenic mediators from KCs and circulating inflammatory cells. Remarkably, ROS directly activate HSCs. The elevated oxidative stress contributes to fibrogenesis via stimulating collagen production from activated HSCs and release of other pro-fibrogenic cytokines and growth factors [126, 128].

## 5. Role of microRNAs (miRNAs) in liver pathophysiology

miRNAs are a family of small non-coding RNAs (20–25 nucleotides in length) that control gene expression by binding to mRNAs to repress translation or induce mRNA cleavage [129]. Many researchers have reported that the unusual expression of miRNAs in liver tissue was related to the pathogenesis of liver disease of any etiology [130, 131]. Recently, miRNAs have been found to play fundamental roles in liver fibrosis, including those in HSC activation and ECM production [132]. For example, miRNA-21 exhibits an important role in the pathogenesis and progression of liver fibrosis. A natural product 3,3′-Diindolylmethane (DIM) inhibits TGF-β signaling pathway by down-regulating the miRNA-21 expression in thioacetamide-induced experimental liver fibrosis. Furthermore, DIM can suppress HSC activation via down-regulating

miRNA-21 levels in HSCs by inhibiting activity of the transcription factor AP-1 [133]. Inhibition of miRNA-21 also reduces liver fibrosis through concomitant reduction of $CD24^+$ liver progenitor cells [134]. In mouse and human studies, the expression levels of miRNA-199a, antisense miRNA-199a*, miRNA-200a, and miRNA-200b are found to be positively and significantly correlated with progression of liver fibrosis. Overexpression of these miRNAs dramatically increases the expression of fibrosis-related genes in HSCs [135]. Also, miRNA-221 and miRNA-222 are up-regulated in human liver in a fibrosis progression-dependent manner [136]. Similarly, in isolated primary human liver cells, miRNA-571 is up-regulated in hepatocytes and HSCs in response to the pro-fibrogenic cytokine TGF-β [137]. miRNA-214 appears to participate in the development of liver fibrosis by modulating the epidermal growth factor (EGF) receptor and TGF-β signaling pathways. Also, inhibition of miRNA-214 by locked nucleic acid-antimiRNA-214 ameliorates liver fibrosis in PDGF c transgenic mice [138]. In addition, miRNA-214-5p may play crucial roles in HSC activation and progression of liver fibrosis. The overexpression of miRNA-214-5p in human stellate cells increases the expression of fibrosis-related genes such as MMP-2, MMP-9, α-SMA, and TGF-β1 [139].

miRNAs may also play anti-fibrogenic roles. It has been demonstrated that both miRNA-150 and miRNA-194 inhibit HSC activation and ECM production in rats with liver fibrosis by decreasing the expression of c-myb (target for miRNA-150) and rac 1 (target for miRNA-194) [140]. Interestingly, miRNAs such as miRNA-19b, miRNA-29, miRNA-133a, and miRNA-146a are significantly down-regulated in HSCs isolated from experimental animals with liver fibrosis, and restoration of these miRNAs alleviates fibrogenesis [47, 141, 142]. Moreover, miRNA-133a overexpression inhibits both human and murine primary HSCs proliferation and prevents the progression of liver fibrosis [142].

Multiple studies have proposed that miRNAs may serve as biomarkers for HSC activation and liver fibrosis progression, and can be possible candidates for future therapies targeting liver fibrosis/cirrhosis.

## 6. Pathogenesis of liver fibrosis/cirrhosis

Liver fibrosis and its end-stage consequence, cirrhosis, represent the final common pathway of almost all chronic liver diseases. Fibrosis and cirrhosis of the liver remain major medical problems with significant morbidity and mortality worldwide. Liver fibrosis is in fact a wound-healing response to liver injury and is characterized by accumulation of fibrotic scar tissue. Although the scar tissue formation is beneficial at first because it encapsulates the injury, the chronic activation of this healing process eventually progresses to advanced fibrosis/cirrhosis. This leads to altered vascular architecture and microcirculation, ischemia, and widespread hepatocyte cell death [143]. Also, in cirrhosis, collagen strands become so prevalent and divide the liver parenchyma into distinct structurally abnormal regenerative nodules, resulting in organ dysfunction [143].

In fact, liver damage leading to cirrhosis is the result of a complex mechanism involving, from direct toxic effects to a sustained inflammatory process, driving to the death of hepatocytes

via apoptosis and liver fibrosis, mediated by secretion of several cytokines [144]. The inflammatory reaction is the coordinated process by which the liver responds to local insults, trying to restore the hepatic architecture and function after acute liver injury [128]. However, if the liver is faced to a sustained local damage, the chronic inflammatory response gives rise to a progressive replacement of healthy liver tissue by non-functional fibrotic scar tissue. The imbalance between tissue regeneration and fibrosis determines the outcome toward health recovery or liver cirrhosis [144].

### 6.1. Imbalance between extracellular matrix synthesis and degradation

Liver fibrosis can be defined as a dynamic and highly integrated molecular, tissue and cellular process regarded as the result of an imbalance between ECM synthesis and degradation. In the healthy liver, ECM is composed of several components such as collagens (mainly the interstitial types I, III, V, VI, and the basement membrane types IV, XV, XVIII, and XIX), glycoproteins (such as laminin isoforms and fibronectin), proteoglycans and elastin [145–147]. Normally, ECM components comprise less than 3% of the relative area of a liver tissue section and approximately 0.5% of the wet weight. During the development of liver fibrosis, there is a 5- to 10-fold increase in the content of collagenous and non-collagenous components, particularly of fibrillar collagen type I and III [146], and an increase of elastin, laminins, and proteoglycans [148]. The total amount of ECM is not only dependent on the rate of synthesis but also largely on the balance between the matrix MMPs, and the TIMPs, especially TIMP-1 [15].

The MMPs are a family of zinc-dependent endopeptidases that can degrade both collagenous and non-collagenous components of ECM in the extracellular space [149]. MMP activity is regulated by TIMPs, which bind to MMPs, blocking their proteolytic activity. The MMPs and TIMPs play a crucial role in the fine regulation of the ECM turnover and the resulting increase in the TIMPs/MMPs ratio in liver promotes fibrosis by protecting accumulated matrix from degradation by MMPs (**Figure 1**) [8].

### 6.2. Mechanisms and mediators of liver fibrogenesis

Liver fibrosis, which is characterized by the excessive deposition of ECM (non-functional connective tissue) components [150], involves both parenchymal and non-parenchymal cells, as well as infiltrating immune cells [151, 152]. Furthermore, several critical signaling pathways have important roles in liver fibrosis. The complex interactions between these signaling pathways and different cells contribute to the progression of liver fibrosis [153].

HSCs are central effectors of fibrogenesis although other cells and processes can make significant contributions. In the healthy liver, HSCs are in a quiescent state with low proliferation rates, store dietary vitamin A, control the ECM synthesis, regulate the local vascular contractility, and serve as the pericytes for the sinusoidal endothelial cells. Damage to hepatocytes activates HSCs transformation into myofibroblast-like cells that play a fundamental role in the development of fibrotic liver response [14]. Myofibroblast-like cells with high proliferative capacity, without vitamin A, exhibit increased expression of $\alpha$-SMA fibers [3]. These cells contribute to fibrosis by producing large amounts of ECM components and collagens (specifically type I) to encapsulate

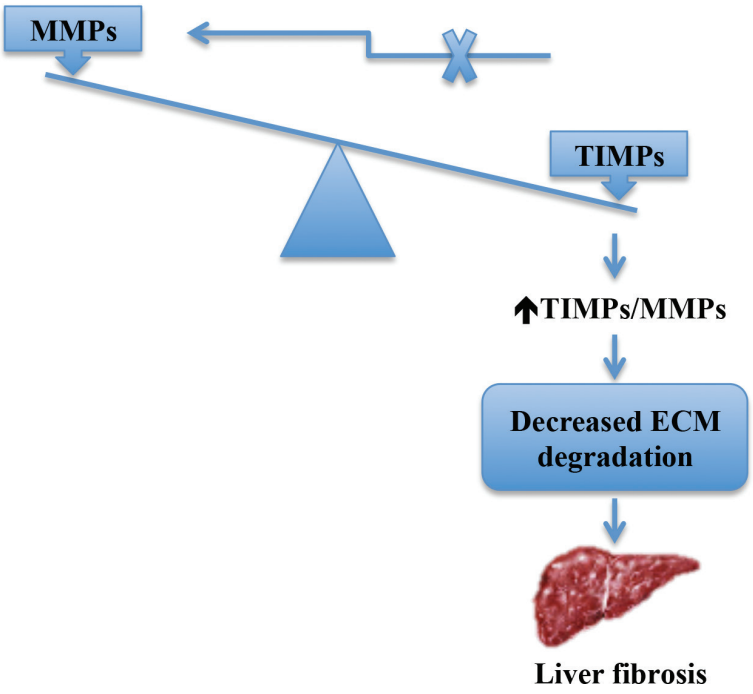


**Figure 1.** Imbalances in ECM synthesis and degradation result in liver fibrosis. Regulation of degradation is determined by the balance between the activity of MMPs and TIMPs. The MMPs degrade both collagenous and non-collagenous components of ECM in the extracellular space. MMP activity is regulated by TIMPs, which bind to MMPs, blocking their proteolytic activity. Increase in the TIMPs/MMPs ratio in liver promotes fibrosis by protecting accumulated matrix from degradation by MMPs. ECM, extracellular matrix; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor of metalloproteinases.

the injury [152]. Although HSCs are classically considered to be a major source of myofibroblasts [154, 155], other cell types like portal myofibroblasts and cells recruited from the bone marrow also contribute to the expansion of the myofibroblast population observed during the liver injury [154]. Activated HSCs also secrete an increased amount of MMPs and their inhibitors, TIMPs, which are necessary for the ECM remodeling [154, 156]. HSC activation leads to the up-regulation of TIMPs and TGF-β1 with the inhibition of MMP activity. The TIMP activation thus stimulates collagen type I synthesis and ECM deposition in the extracellular space [157]. Besides injured hepatocytes, hepatic macrophages (KCs), endothelial cells, and lymphocytes also drive HSC activation [158].

HSC activation is still the primary pathway leading to the liver fibrosis and it consists of two main stages: initiation and perpetuation (**Figure 2**) [126]. The initiation stage is related with the early changes in gene expression and phenotype that render the cells responsive to several cytokines and stimuli. Initiation of HSC activation is stimulated by several soluble factors such as oxidant stress signals (ROS), apoptotic bodies, and paracrine stimuli from neighboring cell types including hepatocytes, KCs, sinusoidal endothelium, and platelets [8, 72]. Hepatocytes

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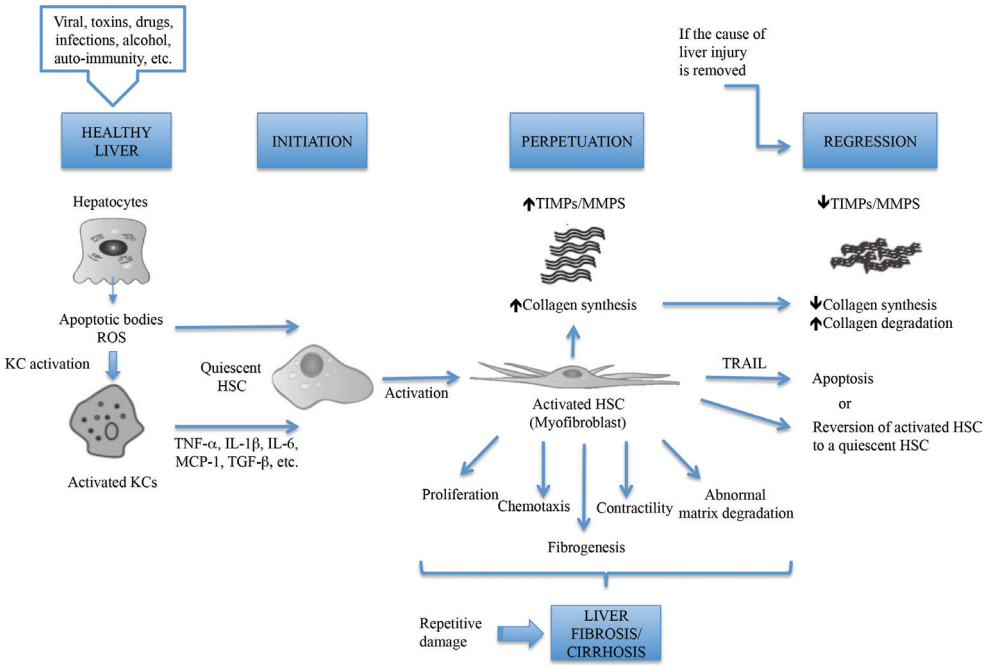


**Figure 2.** Initiation, perpetuation, and regression of liver fibrogenesis involving HSCs. The pathways of HSC activation consist of initiation and perpetuation. Initiation is stimulated by soluble factors such as apoptotic bodies, oxidant stress signals (ROS), and paracrine stimuli from neighboring cell types. Perpetuation includes HSC activation (phenotypic switch from a quiescent type into an activated type) and related cellular changes such as proliferation, chemotaxis, fibrogenesis, contractility, and abnormal matrix degradation. Repetitive damage to liver causes perpetuation of activated HSCs in the liver. Activated HSCs produce excessive collagen, down-regulate release of MMPs and enhance expression of the physiological inhibitors of the MMPs (TIMPs). Imbalances in collagen synthesis and degradation result in liver fibrosis/cirrhosis. During regression, activated HSCs undergo apoptosis or inactivation if the cause of liver injury is removed. ROS, reactive oxygen species; KC, Kupffer cell; HSC, hepatic stellate cell; TNF-α, tumor necrosis factor-α; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; TGF-β, transforming growth factor-β; TIMPs, tissue inhibitor of metalloproteinases; MMPs, matrix metalloproteinases; TRAIL, TNF-related apoptosis-inducing ligand.

are believed to represent a major source of ROS as well as of other oxidative stress-related reactive mediators or intermediates [1]. Hepatocyte apoptosis leads to the release of cellular contents such as DNA and ROS that activate KCs to release pro-inflammatory (such as TNF-α, IL-1β, IL-6, MCP-1) and pro-fibrogenic (especially TGF-β) factors [158]. Hepatocyte apoptosis following injury also promotes initiation of HSC activation through a process mediated by Fas, and this process may involve the TRAIL [159]. After stimulation by cytokines or engulfment of apoptotic bodies, KCs stimulate matrix synthesis and cell proliferation through the actions of cytokines including TGF-β1 and ROS/lipid peroxides [64]. Endothelial cells are also likely to participate by conversion of TGF-β from the latent to the active, pro-fibrogenic form [126]. Platelets are another important source of paracrine stimuli, including PDGF, TGF-β1, and EGF [126]. On the other hand, perpetuation stage results from the effects of these stimuli on maintaining the activated phenotype and generating liver fibrosis. This stage involves

autocrine as well as paracrine cycles. It includes HSC activation and related cellular changes such as proliferation, chemotaxis, fibrogenesis, contractility, and matrix degradation [126]. Activated HSCs proliferate in response to various kinds of cytokines, chemokines, and growth factors such as TGF-β, EGF, and PDGF [2, 8]. TGF-β, which has been identified as the most pro-fibrotic cytokine, promotes expression of collagen type I by activated HSCs and inhibits ECM degradation through the expression of TIMPs [160]. In parallel, PDGF has emerged as the most potent proliferative cytokine for HSCs [8]. Also, activated HSCs show chemotactic response, migrate toward damaged area and start to accumulate [3]. They express the cytoskeleton protein (α-SMA), equipping the cells with a contractile apparatus and collagens (especially type I) [12, 161, 162]. Thus, HSCs are able to constrict individual sinusoids as well as the entire fibrotic liver [3]. The net effect of these changes is to increase ECM deposition. In addition, cytokine release by HSCs can expand the inflammatory and fibrogenic tissue responses, and matrix proteases may hasten the replacement of normal matrix with fibrotic scar [126]. Briefly, activated HSCs are major effectors of liver fibrogenesis by integrating all incoming paracrine or autocrine signals released from both parenchymal and non-parenchymal cells (pro-inflammatory cytokines, growth factors, chemokines, ROS, and others).

Chronic inflammation and fibrosis are inseparably linked and the interactions between immune cells, local fibroblasts and especially subsets of macrophages determine the outcome of liver injury [8]. Macrophage phenotype and function are critical determinants of fibrotic scarring or resolution of injury. Macrophages, which are typically categorized into classically activated (M1) or alternatively activated (M2) phenotypes, play dual roles in the progression and resolution of liver fibrosis [163]. Typically, M1 macrophages play a pro-inflammatory role in liver injury and produce inflammatory cytokines, while M2 macrophages exert an anti-inflammatory role during tissue repair and fibrosis. The imbalance of M1 and M2 macrophages mediates the progression and resolution of liver fibrosis [164]. During the early stages of liver injury, bone marrow-derived monocytes are extensively recruited to the liver and then differentiate into inflammatory macrophages (mostly M1 macrophages) to produce pro-inflammatory and pro-fibrotic cytokines, thereby promoting inflammatory responses and HSC activation. Afterwards, recruited macrophages switch their phenotypes (mostly M2 macrophages) to secrete MMPs for the successful resolution in hepatic scar [153, 165, 166]. Therefore, a complicated interplay between M1 and M2 types of macrophages plays a critical role in fibrogenesis [128].

### 6.3. Liver fibrosis is potentially reversible

Liver fibrosis is thought to be a potentially reversible condition if the cause of liver injury is removed (such as virus suppression or alcohol absence) (**Figure 2**). Regression of liver fibrosis is associated either with elimination of activated HSCs via apoptosis or senescence or with reversion of activated HSCs to a more quiescent phenotype. It has been shown that HSCs are sensitive to Fas and TRAIL-mediated apoptosis, and natural killer cells can induce apoptosis of HSCs by a TRAIL-mediated mechanism [167]. Similarly, TRAIL expressed by KCs is also thought to mediate HSC apoptosis [168]. In addition, apoptosis of activated HSCs is for sure followed by a decrease in collagen production as well as a reduction in TIMP synthesis with an increase in the hepatic MMP expression [1]. Therefore, activated HSCs, the primary source of ECM, are the most attractable target for reversing liver fibrosis [169].

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## 7. Halofuginone

Halofuginone, a non-toxic and low molecular weight plant alkaloid [7-bromo-6-chloro-3-(3-hydroxy-2-piperidine)-2-oxopropyl-4(3H)-quinazoline] (**Figure 3**) isolated from the roots of *Dichroa febrifuga* (Chinese medicinal plant), is used worldwide as an anti-parasitic drug in commercial poultry production [16]. At first, halofuginone was identified as a potent inhibitor of collagen type $\alpha$1 gene expression and ECM deposition. At present, it is being evaluated in clinical trial for Duchenne muscular dystrophy, in which fibrosis is the main complication.

### 7.1. Halofuginone and its effect on collagen synthesis

Halofuginone was found to be a potent inhibitor of collagen type $\alpha$1 gene expression [17], which was demonstrated in a broad range of cell types including rat, mouse, chicken, and human, both *in vitro* and *in vivo* [16–20]. The discovery of the inhibitory effect of halofuginone on collagen synthesis and ECM deposition has led to intensive studies that were aimed to control many diseases associated with excessive collagen accumulation, such as pulmonary, pancreatic and renal fibrosis [21–23], scleroderma and chronic graft-versus-host disease [24], post-operative peritendinous and abdominal adhesions [25, 26], urethral and esophageal strictures [27, 28], wound repair [29], burn injury [30], renal injury [31, 32], injury-induced arterial intimal hyperplasia [33], colitis [34], and liver fibrosis and cirrhosis [35–39]. Inhibition is independent of the route of administration (intraperitoneally, administered locally, or given orally).

Halofuginone was found to inhibit collagen type I synthesis but not that of type II [17] or III [170] *in vitro*. The inhibitor effect of halofuginone on collagen $\alpha$1 synthesis appears not to be a direct effect but rather dependent on new protein synthesis, because concurrent treatment of fibroblasts with protein synthesis inhibitors blocks the suppressive effect of halofuginone on collagen $\alpha$1 mRNA gene expression [18].

Because of the significant impact of fibrosis on human health, there is an unmet need for safe and effective therapies that directly target fibrosis. In animal models of fibrosis, regardless of the tissue, halofuginone had a minimal effect on collagen levels in the control (non-fibrotic) animals; however, it displayed a strong inhibitory effect in the fibrotic organs. This suggests that the regulation of the low-level expression of collagen type I genes differs from that of the

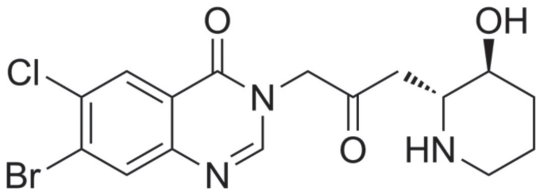


**Figure 3.** Chemical structure of halofuginone.

overexpression induced by the fibrogenic stimulus, which is usually an aggressive and rapid process [171]. Halofuginone mainly affects the stimulated collagen synthesis, therefore, when it is administered systemically, it is actually targeted to the desired fibrotic location without affecting collagen synthesis in other regions.

### 7.2. Halofuginone and TGF-β pathway

TGF-β is a "master switch" in chronic liver disease, being involved in all stages of the disease progression, from initial liver injury, inflammation, fibrosis, to cirrhosis and hepatocellular carcinoma at the end [172]. TGF-β signals through transmembrane receptor serine/threonine kinases to activate novel signaling intermediates called Smad proteins, which then modulate transcription of target genes [173]. TGF-β, signaling via Smad3, is the most pro-fibrogenic cytokine present in the liver and the major promoter of ECM synthesis [173, 174]. It induces pro-fibrotic cellular and transcriptional responses such as induction of the synthesis of ECM components, especially collagen, as well as fibronectin and laminin, and it inhibits the matrix degradation enzymes [175]. In various experimental fibrotic models, no effect of halofuginone was observed on the expression of the TGF-β receptors gene or on TGF-β levels [176–178]. This finding supports the hypothesis that the halofuginone target is down-stream in the TGF-β pathway. Halofuginone is an inhibitor of Smad3 phosphorylation down-stream of the TGF-β signaling pathway [177, 179, 180]. In chemically induced liver fibrosis, halofuginone affects TGF-β regulated genes through inhibition of Smad3 phosphorylation of activated HSCs [181]. It inhibits TGF-β-induced phosphorylation of Smad3 and also increases the expression of the inhibitory Smad7 in several cell types (such as fibroblasts, hepatic and pancreatic stellate cells, tumor cells and myoblasts) [178, 181–183]. The inhibition of Smad3 phosphorylation is associated with the halofuginone-dependent activation of Akt MAPK/ERK and p38 MAPK phosphorylation [182]. Thus, drugs that selectively target individual signaling pathways down-stream of the TGF-β receptor are likely to be more successful.

### 7.3. Halofuginone affects pre-existing fibrosis

Halofuginone affects fibrosis as a preventive agent when it was administered before or together with the fibrotic stimulus [21, 26, 27, 35, 184]. It can elicit resolution of established fibrosis, a capability that sets it apart from all other preventive anti-fibrotic agents. For example, in rats with established thioacetamide-induced liver fibrosis, addition of halofuginone to the diet results in almost complete resolution of the fibrotic condition as measured by hydroxyproline levels in the liver [36]. This is probably due to up-regulation of the collagen degradation pathway by inhibition of the TIMP-1, and activation of MMPs [43]. In addition, halofuginone given orally before fibrosis induction prevents the activation of most of the stellate cells and the remaining cells expressed low levels of collagen $\alpha$1 gene, resulting in low levels of collagen [36]. Furthermore, halofuginone administration in low concentrations prior to and following partial hepatectomy in cirrhotic rats does not inhibit normal liver regeneration, despite the reduced levels of collagen type I mRNA [37]. When given to rats with established fibrosis, halofuginone causes significant reductions in $\alpha$-SMA, TIMP-2, collagen type I gene expression, and collagen accumulation [37]. These animals demonstrate improved capacity for regeneration, suggesting the possible beneficial use of halofuginone before and during fibrotic/cirrhotic liver regeneration.

### 7.4. Halofuginone as an anti-fibrotic agent

In recent years, much attention was focused on halofuginone against liver fibrosis (**Table 3**). Although the exact anti-fibrotic mechanism of halofuginone is not well understood, it is found to be associated with inhibition of TGF-β signaling [179], which is known to inhibit mesengial

| Models | Effects | Mechanisms | References |
|---|---|---|---|
| DMN-induced liver fibrosis/ cirrhosis in rats | Prevents liver cirrhosis | Prevents increase in collagen type I gene expression | [35] |
| TAA-induced liver fibrosis in rats | Causes almost complete resolution of fibrosis | Reduces collagen levels, collagen α1(I) gene expression, TIMP-2 content, and SMA-positive cells | [36] |
| TAA-induced liver cirrhosis in rats | Improves liver regeneration | Reduces α-SMA, TIMP-2, collagen type I gene expression, and collagen accumulation | [37] |
| ConA-induced liver fibrosis in rats | Prevents liver fibrosis | Decreases Th17 cell differentiation and its related cytokines production | [38] |
| ConA-induced liver fibrosis in rats | Attenuates liver fibrosis | Suppresses synthesis of collagen 1, α-SMA and TIMP-2; down-regulates TGF-β1/Smad3 signaling pathway; decreases pro-inflammatory cytokines | [39] |
| TAA-induced liver fibrosis in rats | Up-regulates MMP-3 and -13 and down-regulates TIMP-1 (*in vivo*); inhibits HSC proliferation and migration (*in vitro*) | Activates p38 MAPK and NF-κB | [43] |
| TAA-induced liver fibrosis in rats | Inhibits HSC activation and collagen synthesis; prevents activation of TGF-β-dependent genes | Inhibits Smad3 phosphorylation | [181] |
| TAA-induced liver fibrosis in rats | Affects cross-talk between hepatocytes and HSCs | Up-regulates synthesis and secretion of IGFBP-1 | [192] |
| TAA-induced liver fibrosis in rats | Prevents liver fibrosis and improves cirrhotic liver regeneration | Increases expression of early genes of regeneration (PRL-1 and IGFBP-1) | [193] |
| Human hepatoma cell injected mice | Suppresses tumor growth | Increases IFN-γ and IL-2 | [196] |
| Diethylnitrosamine and *N*-nitrosomorpholine-induced HCC in rats | Suppresses lung metastasis | Inhibits MMP | [197] |

*Abbreviations*: DMN, dimethylnitrosamine; TAA, thioacetamide; TIMP, tissue inhibitor of metalloproteinase; SMA, smooth muscle actin; ConA, Concanavalin A; Th17, T helper 17; TGF-β, transforming growth factor-β; MMP, matrix metalloproteinase; HSC, hepatic stellate cell; p38 MAPK, p38 mitogen-activated protein kinase; NF-κB, nuclear factor-κB; IGFBP-1, insulin-like growth factor binding protein-1; PRL-1, tyrosine phosphatase; IFN-γ, interferon-γ; IL-2, interleukin-2; HCC, hepatocellular carcinoma.

**Table 3.** Effects of halofuginone in various experimental liver diseases.

cell proliferation and ECM deposition [185]. In several animal models of fibrosis, in which excess collagen is the characteristic of the disease, halofuginone prevents transition of the fibroblasts to myofibroblasts by inhibition of Smad3 phosphorylation down-stream of the TGF-β signaling pathway [186, 187], thereby inhibits collagen synthesis [186]. Halofuginone also regulates cell growth and differentiation, apoptosis, cell migration, and immune cell function [41]. It prevents concanavalin A-induced liver fibrosis by affecting Th17 cell differentiation, which suggests a direct link between the myofibroblasts/fibrosis pathway and the Th17 pro-inflammatory pathway [38]. Th17 cells, a distinct subset of $CD4^+$ T cells with IL-17 as their major cytokine, orchestrate the pathogenesis of inflammation [171]. It has been suggested that halofuginone-dependent inhibition of fibrosis includes selective inhibition of the Th17 cell development by activating the amino acid starvation response [188, 189]. Halofuginone activates the amino acid starvation response by directly inhibiting the prolyl-tRNA synthetase activity of glutamyl-prolyl-tRNA synthetase [190]. Furthermore, addition of exogenous proline reverses a broad range of halofuginone-induced cellular effects, indicating that glutamyl-prolyl-tRNA synthetase-inhibition underlies the therapeutic activities of halofuginone [190]. TGF-β is required for facilitation of differentiation of the inflammatory Th17 cell subset [191], which suggests the presence of a connection between the TGF-β signaling inhibition and the amino acid starvation response [187]. Treatment with halofuginone also effectively inhibits the delayed-type hypersensitivity response, indicating suppression of T cell–mediated inflammation *in vivo* [42]. Moreover, it was shown that halofuginone is a potent inhibitor of NF-κB, pro-inflammatory cytokines, and p38 MAPK phosphorylation in activated T cells *in vitro* [42]. Also, submicromolar concentrations of halofuginone inhibit HSC proliferation and migration and up-regulate their expression of fibrolytic MMP-3 and -13 via activation of p38 MAPK and NF-κB. The remarkable induction of MMP-3 and -13 makes halofuginone a promising agent for anti-fibrotic combination therapies [43]. Halofuginone also affects the cross-talk between the hepatocytes and the HSCs by up-regulating the synthesis and secretion of insulin-like growth factor binding protein-1 (IGFBP-1), which inhibits HSC migration [192]. It also affects the expression of early genes of liver regeneration, IGFBP-1 whose synthesis and secretion is regulated in part by TGF-β [192] and tyrosine phosphatase (PRL-1) whose synthesis is regulated by transcription factor early growth response-1 (Egr-1) probably via TGF-β [193].

### 7.5. Anti-tumoral role of halofuginone

In many types of tumor, there is a strong relationship between tissue fibrosis and increased risk of tumor development. For example, the leading risk factor for hepatocellular carcinoma is liver cirrhosis, and its associated inflammation, regeneration, and fibrosis [194, 195]. Tumor cells develop and metastasize more effectively in fibrotic tissues; therefore, any reduction in tissue fibrosis reduces the risk of cancer [171]. Halofuginone reduces tumor growth and mortality in xenograph mice implanted with human hepatoma cells [196]. In diethylnitrosamine and *N*-nitrosomorpholine-induced, spontaneously metastasizing hepatocellular carcinoma, halofuginone suppresses lung metastasis in rats through MMP inhibition [197]. Moreover, halofuginone treatment results in effective inhibitory effects on the cascade of events leading to angiogenesis (formation of new blood vessels), such as abrogation of endothelial cell MMP-2 expression, basement membrane invasion, capillary tube formation, vascular sprouting, and

deposition of sub-endothelial ECM *in vitro* [171]. Inhibition of angiogenesis is mostly accompanied by inhibition of the fibroblasts to myofibroblasts transition, reduction in tumor stroma ECM, and inhibition of tumor growth [171]. The high effectiveness of halofuginone in reducing fibrosis, which affects tumor growth and tissue regeneration in the liver, arises from its dual role in inhibiting the TGF-β signaling and Th17 cell development [187].

## 8. Conclusion

Fibrosis is a pathological process associated with excessive ECM deposition that leads to destruction of organ architecture and function. Fibrosis contributes enormously to deaths worldwide; thus, effective therapies are of a great need. Halofuginone has great potential as an anti-fibrotic therapeutic. Systemic administration of halofuginone in animal models and humans is well tolerated [24]. Additionally, in most animal models of fibrosis, halofuginone has a minimal effect on collagen levels in non-fibrotic animals, while exerting strong inhibitory effects in fibrotic organs. It mainly affects stimulated collagen synthesis without altering the usual low physiological level of collagen expression. Because halofuginone inhibits collagen type I synthesis on the transcriptional level and reduces ECM deposition, it is a promising candidate for treatment of diseases associated with excessive ECM, such as liver fibrosis/cirrhosis. Thus, halofuginone meets the criteria as a promising anti-fibrotic drug for further evaluation in the treatment of liver fibrosis/cirrhosis.

**Conflicts of Interest**

The author reports no conflicts of interest.

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