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## **Liver fibrosis: the 2017 state of art**

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## **Abstract**

Liver fibrosis is a wound-healing response to a wide spectrum of chronic liver injuries. It is characterized by loss of hepatocytes and alteration in hepatic architecture following an imbalance between extracellular matrix synthesis and degradation. Irrespectively of underlying etiology, fibrosis may progress to cirrhosis and specific pathogenetic mechanisms as well as different disease patterns may be identified according to etiology.

Liver biopsy is still considered the gold standard for fibrosis assessment, despite the fact that it is invasive, has poor patient compliance and is not exempt of complications. Several reliable and non-invasive tools are currently used in clinical practice, including imaging methods and surrogate serum biomarkers, commonly combined into composite scores. The main limitation of non-invasive methods is the low performance in the discrimination of intermediate stages of fibrosis. However, with the recent availability of novel treatment options, particularly for chronic hepatitis C, a precise staging of liver fibrosis is becoming clinically less relevant. Conversely, since patients with cirrhosis need to be monitored for the risk of hepatocellular carcinoma development, the accurate detection of this condition is a primary endpoint.

Finally, several promising antifibrotic agents are under investigation in phase I and II trials.

Nevertheless, further efforts are needed for the identification of novel potential targets for the development of antifibrotic drugs able to arrest, and possibly revert liver fibrogenesis.

**Key words:** chronic hepatitis - extracellular matrix - hepatic stellate cells - liver fibrosis

The natural history of chronic hepatitis is characterized by long-lasting inflammation and hepatocellular necrosis that lead to fibrogenesis, which overtime progresses in a variable percentage of patients to cirrhosis. Prognosis and clinical management of patients with chronic hepatitis are strongly affected by the degree of liver fibrosis. Thus, accurate staging of fibrosis is crucial to assess the risk of evolution towards cirrhosis and its complications such as hepatocellular carcinoma (HCC) and liver failure both in viral and non-viral chronic liver diseases.<sup>1, 2</sup>

Worldwide, the number of chronic hepatitis C virus (HCV) infected persons is estimated to be around 185 million, whereas approximately 248 million individuals are chronically infected with hepatitis B virus (HBV) despite decades of vaccination.<sup>3-5</sup> Remarkably, in Western Countries, non-alcoholic fatty liver disease (NAFLD) is becoming the most common liver disorder affecting 17%-46% of adults,<sup>6</sup> and its progressive form (non-alcoholic steatohepatitis or NASH) accounts for 7%-30% of NAFLD patients.<sup>7</sup>

Across the wide spectrum of liver diseases, long-term hepatic necro-inflammation is the common feature triggering liver fibrosis development and progression.<sup>8</sup> However, several aspects related to etiology, genetics and epigenetics could impact pathogenesis, diagnosis and treatment.<sup>9-14</sup> In this paper, we focus on viral and metabolic types, and review the mechanisms of liver fibrogenesis, the currently available diagnostic tools and the potential novel therapies.

### **Pathogenesis of liver fibrosis**

Liver fibrosis is a wound-healing response to a wide spectrum of chronic liver injuries and results from an imbalance between extracellular matrix (ECM) synthesis and degradation.<sup>15</sup> Normally, ECM is composed of fibrillar collagens type I and III, and microfibrillar collagens type IV. Excessive ECM deposition, especially fibrillary collagens, causes an alteration of the normal hepatic architecture with progressive substitution of the liver parenchyma with scar tissue.<sup>16</sup>

The key factor of fibrogenesis is the activation and proliferation of myofibroblasts, with hepatic stellate cells (HSCs) being the major contributors of myofibroblast pool (82-96%).<sup>17</sup> HSCs are non-parenchymal quiescent cells residing in the space of Disse that normally account for 5-8% of total liver cells.<sup>18</sup> The main functions of HSCs include vitamin A storage, sinusoidal blood flow regulation through endothelial cell interactions, xenobiotic detoxification, immune-tolerance regulation and ECM homeostasis.<sup>19</sup> However, following pathological insults with consequent release of pro-fibrogenic cytokines and various growth factors, HSCs switch from a normal quiescent state to an activated myofibroblast phenotype.<sup>20</sup> HSCs activation leads to loss of balance between matrix metalloproteinases (MMPs), which are zinc-dependent ECM degrading enzyme, and tissue inhibitors of metalloproteinase family (TIMPs), resulting in inhibition of ECM degradation.<sup>21</sup> However, a disease specific pattern could be identified in fibrosis development. Chronic viral hepatitis is characterized by porto-central septa and interface hepatitis whereas NASH is distinguished by intercellular fibrosis and fibrillar ECM deposition around sinusoids.<sup>22</sup>

### **Liver fibrosis in chronic hepatitis B**

The host immune system is tightly involved in liver disease pathogenesis.<sup>23</sup> Besides inflammation, different viral factors are associated to viral hepatitis-related fibrosis and cirrhosis, particularly in chronic HBV infected patients in whom viral genotype and viral replication are the main factors that contribute to fibrosis progression.<sup>24, 25</sup> In Asiatic populations, it has been reported that HBV genotype C is associated with more severe liver disease.<sup>26</sup> Kao *et al.*, in Taiwanese chronic hepatitis B (CHB) patients, investigating the relationship between HBV genotypes and liver disease severity, found that genotype C was prevalent in patients with cirrhosis (60% vs. 23%,  $p < 0.001$ ).<sup>24</sup> Accordingly, Sumi *et al.* reported that among 258 patients with histologically verified chronic liver disease, the ratio

of patients with advanced fibrosis in genotype C was significantly higher than that in genotype B (22/74 vs. 30/40, respectively;  $p=0.034$ ).<sup>27</sup> On the contrary, a retrospective study involving 262 Caucasian patients with chronic HBV infection (prevalent genotypes D and A, 27% and 24%, respectively) failed to demonstrate an association between a given HBV genotype and liver disease severity.<sup>28</sup>

Beside HBV genotype, basal core promoter (BCP) mutations have been associated to liver disease progression. In particular, a sequential accumulation of BCP mutations have been reported during CHB natural history, with A1762T/G1764A mutations selected in the early course of infection, G1986A pre-core mutation during liver disease progression and mutations at nucleotides 1753 and 1989 in the late course of chronic liver disease, with prevalence in patients with cirrhosis.<sup>29</sup> Similarly, Chu *et al.* found that A1762T/G1764A BCP mutant was associated with about 4-fold increased risk of cirrhosis<sup>30</sup> and, more recently, Tseng *et al.* reported that A1762T/G1764A BCP mutant percentage > 45% was associated with an increased risk of cirrhosis development (adjusted odds ratio [OR]=2.81; 95% confidence interval [CI]: 1.40-5.67).<sup>31</sup> Interestingly, it has been indicated that such mutation affects codons 130 and 131 of the X gene (K130M and V131I) thus altering HBx protein and contributing to HCC development.<sup>32</sup> In addition, it has been reported that HBx protein could activate HSCs *in vitro* through paracrine secretion of transforming growth factor (TGF)- $\beta$ , a cytokine involved in fibrogenesis.<sup>32</sup> Moreover, HSCs exposed to conditioned medium from HBx-expressing hepatocytes showed increased expression of fibrillar collagen type I, connective tissue growth factor (CTGF),  $\alpha$  smooth muscle actin ( $\alpha$ SMA) and MMP-2.<sup>33</sup> Accordingly, Guo *et al.*, investigating HBx effects on the proliferation and expression of fibrosis-related molecules in the human HSCs line (LX-2), found that HBx accelerated G1-S progression in LX-2 cells and that  $\alpha$ SMA, TGF- $\beta$ 1, TGF- $\beta$ RII, CTGF and

collagen I expression was significantly increased in the co-cultures of LX-2 cells with stable HBx transfected cell line.<sup>34</sup>

Finally, viral replication plays a significant role in HBV-related liver fibrogenesis.<sup>35, 36</sup> A population-based prospective cohort study, of 3582 untreated HBV-infected patients prevalently hepatitis B e antigen (HBeAg)-negative (3073/3582; 85.8%), showed that cumulative incidence of cirrhosis increased with HBV DNA level, ranging from 4.5% in patients with HBV DNA <300 copies/mL to 36.2% in those with HBV DNA  $\geq 10^6$  copies/mL ( $p < 0.001$ ).<sup>24</sup> In addition, the risk for cirrhosis was independent on HBeAg *status* and serum alanine aminotransferase (ALT) level.<sup>24</sup> However, two independent studies reported that in HBeAg-positive subjects, HBV DNA levels were inversely correlated to fibrosis degree,<sup>37</sup> and that older age (OR=1.049; 95%CI: 1.017–1.083,  $p=0.003$ ), elevated ALT (OR=0.766; 95%CI: 0.551–0.993,  $p=0.044$ ), and lower HBV DNA levels (OR=1.011; 95%CI: 1.004–1.018,  $p=0.003$ ) are independently associated with significant fibrosis.<sup>38</sup> Likely, in immune-reactive patients, an extensive immune-mediated response towards HBV may lead to both viral load suppression and liver inflammation resulting in hepatitis progression. Indeed, patients in immune-tolerant phase of HBV infection, exhibit high viral load but no inflammation, and consequently fibrosis.

### **Liver fibrosis in chronic hepatitis C**

In chronic hepatitis C (CHC), immunological response is the major factor associated to liver disease progression. However, structural (core, E2) and non-structural (NS3 and NS5) HCV proteins may directly contribute to liver fibrogenesis through different pathways.<sup>39</sup> It has been showed that HCV core protein could upregulate TGF- $\beta$ 1 *in vitro*.<sup>40</sup> Both core protein and NS3 could induce TGF- $\beta$ 1 expression through the generation of reactive oxygen species (ROS) and activation of p38 MAPK,

JNK, ERK1/2, NFκB-dependent pathways or stimulating the secretion of pro-inflammatory chemokines such as interleukin (IL)-8, MCP-1, and RANTES that may participate in inflammatory cells recruitment.<sup>41, 42</sup> Recently, Li *et al* found that HCV NS5A-transactivated protein 13 (NS5ATP13) was upregulated in fibrotic liver tissues and was able to enhance ECM production and human HSCs activation, hence acting as a profibrogenic factor.<sup>43</sup> The E2 glycoprotein of HCV envelope binding to CD81 of HSCs, induces a time-dependent increase in the synthesis and activity of MMP-2. Consequently, the increased ECM degradation may favor inflammatory infiltration and further parenchymal damage.<sup>44</sup>

Unlike HBV viral factors, the role of HCV genotypes and HCV RNA levels in liver disease progression is marginal. Both genotype 1 and high viral load were predictor of non-response to interferon (IFN)-based treatment regimens, thus indirectly associated to liver disease progression.<sup>45, 46</sup> Conversely, it has been shown that different host factors including age of infection older than 40 years, alcohol consumption  $\geq 50$ g/day, and male sex have a stronger association with fibrosis progression than virological factors in HCV infection.<sup>47</sup> In addition, different host genetic variants are associated to a more rapid fibrosis progression.<sup>48</sup> In particular, Tamaki *et al* investigated the genetic risk factors associated with fibrosis progression by analyzing 176 CHC patients who did not achieve sustained virologic response to IFN-based therapy.<sup>49</sup> The authors found a significant fibrosis progression rate in IL28B (rs8099917) TG/GG and *PNPLA3* (rs738409) CG/GG carriers and both genotypes were independent predictors of rapid fibrosis progression at multivariate analysis (IL28B TG/GG hazard ratio [HR]: 3.9, p=0.001, and *PNPLA3* CG/GG HR: 3.1, p=0.040).<sup>49</sup> Similarly, Ali *et al* found that *PNPLA3* CG/GG was significantly associated with the presence of cirrhosis (OR: 1.76; p<0.05) in a large cohort (n=937) of CHC patients.<sup>50</sup>



Finally, a recent study reported that differences in ECM turnover could reflect an etiology-specific ECM composition during fibrogenesis in both CHB and CHC infections.<sup>51</sup> Interestingly, the basement membrane biomarkers P4NP7s (marker of collagen type IV formation) and C4M (marker of MMP-degraded type IV collagen) were significantly elevated in CHB patients, whereas pro-C3 (marker of collagen type III formation) was increased in CHC patients,<sup>51</sup> suggesting that collagen deposition and remodeling depend on the type of viral insult, thus harboring potentially relevant pathogenetic and diagnostic implications.

### **Liver fibrosis in non-alcoholic fatty liver disease**

NAFLD is a common cause of chronic liver disease strictly linked to obesity and diabetes. Most patients with NAFLD have simple fatty liver (NAFL), a benign and reversible condition that in a subset of patients may progress to a more severe form, NASH, characterized by the joint presence of steatosis, inflammation, ballooning degeneration with or without the presence of fibrosis. Mechanisms involved in the pathogenesis of NASH and in the development of fibrosis are poorly understood.

The pathogenesis of NASH has been considered for a long time a “two hit” process.<sup>52</sup> The first hit is the development of hepatic steatosis through the accumulation of triglycerides in hepatocytes, while the second hit includes a variety of inflammatory processes, such as oxidative stress, apoptosis, gut-derived stimulation and the release of pro-inflammatory cytokines from the adipose tissue that can potentially promote hepatic fibrogenesis.<sup>53</sup> However, a recent genome-wide association study (GWAS) identified *PNPLA3* as a key gene in the development of NASH indicating that genetic background could significantly impact on the progression of the liver disease.<sup>54</sup> Particularly, the single nucleotide polymorphism (SNP) rs738409\_G risk allele, that is associated with both hepatic inflammation and steatosis, could explain why some populations are more prone to develop NASH. For example,

Hispanics show a higher (45%) prevalence of steatosis compared to European-Americans (33%) and African-Americans (24%). Furthermore, Hispanics show also a higher prevalence of NASH and cirrhosis while African-Americans are less predisposed to develop liver failure.<sup>54</sup>

### *Lipotoxicity and fibrogenesis*

In the setting of NAFLD, fibrosis may develop independently of inflammation due to the direct pro-fibrogenic action of ROS.<sup>55</sup> In NAFLD, ROS generation may derive from an altered metabolic state. Briefly, the impaired lipolysis in the adipose tissue caused by insulin resistance (IR), leads to an excessive release of free fatty acids (FFAs) that reach the liver. In the hepatocytes, FFAs surplus triggers lipoperoxidation thus favoring the development of ROS and their reactive intermediates, such as 4-hydroxy-2,3-nonenal (HNE), by the mitochondrial electron transport chain or other redox enzymes. Toxic lipids are able to activate several cellular stress pathways contributing to the endoplasmic reticulum (ER) stress, which is one of the most important factor for disease progression in patients with NASH.<sup>56,57</sup>

Furthermore, it has been reported that ROS, together with oxidized LDL, may activate HSCs thus promoting inflammation and fibrosis.<sup>58</sup> Particularly, HNE is able to up-regulate the expression of several profibrogenic genes including pro-collagen type I, TIMP-1 and monocyte chemotactic protein 1 (MCP-1 or CCL2) probably through the c-Jun N-terminal kinase (JNKs), activator protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) molecular pathways.<sup>59</sup>

### *Apoptosis and fibrogenesis*

Excessive oxidative stress induced by ROS results in apoptosis, a mechanism that contribute to the progression of different liver diseases.<sup>60-62</sup> Inflammation, regeneration and fibrosis may all be

promoted by apoptosis of adjacent cells.<sup>63</sup> Particularly, hepatic fibrosis has the potential to favor the development of cirrhosis, and eventually, HCC.<sup>16</sup> Studies have shown that attenuation of hepatic apoptosis was able to reduce fibrogenesis while the blockage of the anti-apoptotic member of the B-cell lymphoma-2 (Bcl-2) family enhanced it.<sup>64, 65</sup> The engulfment of apoptotic bodies by HSCs promotes fibrogenesis through the activation of nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2), the oxidase system in the phagocytes.<sup>64, 66</sup> Activated HSCs also secrete MMPs mediating the release of tumor necrosis factor (TNF)- $\alpha$  in the blood, in turn activating other MMPs in a feed-forward damage response.<sup>67</sup>

#### *Gut-derived stimulation and fibrogenesis*

A large number of microbial species reside in the gastrointestinal tract, and gut microbiota dysbiosis is associated with diseases ranging from localized gastroenterologic disorders to neurologic, respiratory, metabolic, hepatic, and cardiovascular illnesses.<sup>68</sup>

Gut microbiota is implicated in the pathogenesis and progression of NAFLD, through the so-called gut-liver axis.<sup>69</sup> Pathogen associated molecular patterns (PAMPs) (lipoproteins, bacterial DNA, double-stranded RNA), seem to play an important role in fibrogenesis through their interaction with toll-like receptors (TLR)s, on fibroblasts.<sup>70</sup> This interaction promotes the differentiation of these cells into collagen-producing myofibroblasts in turn establishing a proinflammatory/profibrogenic condition leading to the activation of HSCs-expressing TLRs.<sup>71</sup> In addition, microbiota changes may increase the intestinal permeability favoring the propagation of inflammatory signal from the gut to the portal circulation and the liver. The altered intestinal permeability in mouse models has been associated to oxidative stress, ER stress and gut-derived lipopolysaccharide (LPS), and seems to trigger inflammatory responses and progressive liver damage.<sup>72</sup> Patients with NAFLD show increased

intestinal permeability due to the disruption of intercellular tight junctions in the intestine where the bacterial overgrowth is increased.<sup>73</sup>

Recently, several studies established a link between the gut-liver axis and HCC development through the increase in deoxycholic acid, a gut bacterial metabolite able to damage DNA and to promote hepatocarcinogenesis.<sup>74</sup>

### *Role of cytokines in fibrogenesis*

Several lines of evidence have shown that adipokines can be considered as active modulators of hepatic fibrogenesis due to their different expression in healthy subjects compared to those with metabolic abnormalities such as IR or diabetes. Moreover, obesity and IR are significantly associated with fibrosis in different chronic liver diseases.<sup>75</sup>

Adiponectin showed hepato-protective and anti-fibrogenic effects in mice models of alcoholic steatohepatitis (ASH) and NASH.<sup>76</sup> In the setting of NAFLD/NASH, pericellular fibrosis was more severe in adiponectin-deficient mice fed high fat diet compared to wild type mice.<sup>77</sup> Furthermore, adiponectin may delay the progression of experimental NASH from cirrhosis to HCC.<sup>78</sup> This anti-fibrogenic effect is mainly due to the direct interaction of adiponectin with HSCs through specific receptors that in turn promote HSCs apoptosis.<sup>79, 80</sup>

On the contrary, leptin shows a pro-fibrogenic effect through its action on HSCs, Kupffer cells (KC) and sinusoidal cells. Leptin up-regulates type I procollagen, enhances TGF- $\beta$  pathway, induces the expression of TIMP-1, and stimulates HSCs proliferation and survival.<sup>81-83</sup> In addition, leptin may stimulate the phagocytic activity of and cytokines release (for example TGF- $\beta$ ) by KC that in turn indirectly activate HSCs.<sup>84</sup>

Another cytokine that seems to be important in fibrogenesis is resistin even if its biological role is controversial.<sup>85</sup> Resistin is expressed only in quiescent HSCs in mice, while in human, recombinant resistin up-regulates the expression of MCP-1 and IL-8 through the NF-kB pathway.<sup>86</sup>

#### *Role of genetics factors in fibrogenesis*

The *PNPLA3* rs738409 C>G polymorphism is considered one of the main genetic risk factors involved in the development of NAFLD and its progression. *PNPLA3* is expressed in the liver by both hepatocytes and HSCs where it is regulated by insulin levels (through the induction of sterol regulatory element binding protein-1c [SREBP-1c] and carbohydrate response element binding protein [ChREBP]) and by retinol levels, respectively.<sup>87</sup> In both hepatocytes and HSCs, *PNPLA3* protein is located in the membrane of lipid droplets and showed an hydrolyse activity against triglycerides and retinyl esters.<sup>87-89</sup> The rs738409 polymorphism results in loss of function of the protein with liver fat retention in hepatocytes and retinol retention in HSCs. The latter modifies HSCs phenotype from quiescent retinol depot to activated myofibroblasts which begin to secrete collagen. In addition, the release of retinol from HSCs in turn contributes to their activation.<sup>90</sup>

### **Diagnosis of liver fibrosis**

#### *Liver biopsy*

Traditionally, liver biopsy was the only available tool enabling the assessment of liver fibrosis and inflammation, further providing additional information regarding steatosis and unexpected cofactors or comorbidities. To investigate inflammation activity grade and to stage the amount and type of liver fibrosis, many scoring systems have been proposed.<sup>1</sup>

Liver biopsy is still considered the gold standard for fibrosis assessment, despite several limitations and risks that make it unsuitable for patients' monitoring.<sup>91</sup> With the novel accurate non-

invasive tools, in the setting of chronic viral hepatitis, liver biopsy is performed only in case of contradictory results with non-invasive markers.<sup>92,93</sup> In addition, with the occurrence of direct acting antivirals (DAAs) for CHC that safely allow treatment even in patients with advanced cirrhosis, the detection and the accurate staging of liver fibrosis have become clinically less relevant.

Conversely, liver biopsy is essential for the differentiation of simple fatty liver from NASH.<sup>94</sup> Indeed, the diagnosis of NASH requires the simultaneous presence of steatosis, lobular inflammation and ballooning.<sup>95</sup>

### *Imaging methods*

Different imaging methods have been developed to non-invasively assess the extent of liver fibrosis. However, all available methods rely on the measurement of liver stiffness (LS), which is an intrinsic property of the liver parenchyma. Among available methods, the most widely used and validated technique is transient elastography (TE) (Fibroscan<sup>®</sup>, Echosense, Paris, France),<sup>1,96</sup> that allows fibrosis assessment by measuring the velocity of an elastic shear wave propagation through the liver.<sup>97</sup> Harder the liver tissue, faster the shear wave propagates.<sup>98</sup> Liver stiffness measurement is expressed in KiloPascals (kPa), with ranges between 2.5 to 75 kPa, and related to the METAVIR score (F0-F4). The principal advantages of TE include good reproducibility and high performance for cirrhosis detection (area under the curve [AUC]>0.9), as reported by a large European study involving 1257 patients with chronic liver disease.<sup>99</sup> Conversely, for patients with low or mild fibrosis, TE accuracy is lower.<sup>100</sup> However, importance of TE resides in ruling out advanced fibrosis/cirrhosis rather than exactly defining fibrosis stages. According to liver disease etiology, different cut-offs have been proposed for the diagnosis of cirrhosis: 12.5 kPa in HCV, 11.7 kPa in HBV, 10.3 kPa in NASH, 17.9 kPa in biliary liver diseases and 22.7 kPa in alcoholic liver disease.<sup>101</sup>

Finally, TE is a rapid examination, easy to learn and perform. Boursier *et al* showed that a novice observer can obtain a reliable LS result after a single training session, irrespectively of professional status (novice/expert agreement for TE results varied with LS level: <9 kPa,  $r=0.49$ ;  $\geq 9$  kPa,  $r=0.87$ ).<sup>102</sup> However, unreliable results could be obtained in patients with narrow intercostal spaces, ascites and body mass index (BMI) $>28$  kg/m<sup>2</sup>.<sup>98, 103</sup> Additional confounding factors include ALT flares, extra-hepatic cholestasis, congestive heart failure, excessive alcohol intake and non-fasting patient at the time of examination.<sup>104-108</sup>

Besides TE, several real-time elastographic methods such as acoustic radiation force imaging (ARFI) and supersonic shear wave imaging (SSI) have been recently developed for liver fibrosis assessment.<sup>109</sup>

Similarly to TE, ARFI and SSI provide LS quantitation by measuring the velocity of a local shear wave through liver tissue, but unlike TE, these methods are integrated on standard ultrasonography devices and explore a defined region of interest below liver capsule, free of large vascular structures, that can be adapted by the operator.<sup>110</sup> Both methods show high accuracy for severe fibrosis/cirrhosis detection compared to TE (AUC $\geq 0.9$ ).<sup>111</sup> However, in a study including 332 patients with or without liver disease, a significantly higher percentage of reliable measurements, defined as success rate  $\geq 60\%$  and an interquartile range  $<30\%$ , was obtained using ARFI vs. TE and SSI (92.1% vs. 72.2%,  $p<0.001$  and 92.1% vs. 71.3%,  $p<0.001$ , respectively).<sup>112</sup>

### *Non-invasive biomarkers*

Liver fibrogenesis is a dynamic and continuous process arising from the balance between deposition and removal of ECM. Non-invasive biomarkers include two main categories: direct markers of fibrogenesis, as expression of either deposition or removal of liver ECM, and indirect markers, reflecting alterations in hepatic function induced by fibrosis, without a direct link with fibrogenesis.<sup>113</sup>

Direct markers include hyaluronic acid (HA), laminin, type IV collagen, type II collagen N-terminal peptide, MMPs and cytokines, such as TNF- $\alpha$  and TGF- $\beta$ .<sup>114</sup> Indirect markers are mainly obtained by combining together routinely performed blood tests for the calculation of composite scores such as aminotransferase (AST) to platelet ratio index (APRI), FibroTest<sup>®</sup>, Hepascore<sup>®</sup>, enhanced liver fibrosis (ELF<sup>®</sup>) score and NAFLD fibrosis score (NFS).<sup>96</sup>

Overall, surrogate biomarkers are less accurate in detecting intermediate stages of fibrosis compared to cirrhosis. In addition, it has been shown that the combination of different biomarkers could improve diagnostic accuracy compared to a single marker alone.<sup>115</sup> Rosso *et al.* reported that the combination of cytokeratin18-Aspartate396 and LS improved the performance for the detection of significant and advanced fibrosis in NAFLD/NASH patients compared to CK18-Asp396 or LS alone ( $\Delta$  area under the curve [AUC]=0.033,  $p=0.024$ , and  $\Delta$ AUC=0.046,  $p=0.008$ , respectively).<sup>115</sup> In patients with CHC, the combination of serum type IV collagen, laminin, APRI, and albumin resulted in an AUC value of 0.831 for significant fibrosis, 0.791 for advanced fibrosis, and 0.881 for cirrhosis,<sup>116</sup> whereas in CHB, a combination of peptide ions (serum transferrin, complement component C3c and transferrin) identified by mass spectrometry-based multiple reaction monitoring, showed an AUC of 0.848~0.966 and 0.785~0.875 for the identification of significant fibrosis (S2-S4 vs. S0-S1) and severe fibrosis (S3-S4 vs. S0-S2).<sup>117</sup>

Besides novel proposed biomarkers or scores, APRI, FibroTest<sup>®</sup> and NFS are currently the most widely used and validated tests. Main surrogate serum biomarkers for non-invasive evaluation of liver fibrosis and their performance are reported in Table 1.<sup>113, 118-125</sup> A meta-analysis including 33 studies with 8739 CHC patients, found a summary AUROC for APRI of 0.77, 0.80, and 0.83 for the diagnosis of significant fibrosis, severe fibrosis and cirrhosis respectively,<sup>126</sup> whereas another meta-analysis including 1798 CHB patients reported mean AUROC values of 0.79 and 0.75 for significant fibrosis



and cirrhosis.<sup>127</sup> FibroTest® showed a mean AUC of 0.81 and 0.90 for the detection of significant fibrosis and cirrhosis in patients with CHC (n=1679),<sup>128</sup> and mean AUC of 0.84 and 0.90, respectively, in patients with CHB (n=1640).<sup>129</sup> NFS was created and validated analyzing data of 733 patients with biopsy confirmed NAFLD and showed an AUC of 0.88 and 0.82 for severe fibrosis/cirrhosis detection both in estimation and validation cohort.<sup>123</sup>

## **Therapeutic perspectives**

The primary approach for the treatment of hepatic fibrosis consists, first of all, in the elimination of the cause of liver disease, for example, by virus eradication, alcohol abstinence or through appropriate diet.<sup>130</sup> In the setting of HCV, the therapeutic efficacy of DAA to delay the end-stage complications is under evaluation.<sup>131, 132</sup>

Several phase I and II trials are investigating the effect of different antifibrotic agents with promising results. Emricasan, a pan-caspase inhibitor, can reduce liver fibrosis in bile duct ligated mouse.<sup>133</sup> Furthermore, in both HCV and NASH, Emricasan significantly reduces transaminases levels.<sup>134,135</sup> Galectins are cell-surface glycoproteins involved in the regulation of cell migration and inflammatory signaling. Galectin inhibitors, such as GR-MD-02, have shown good safety and tolerability, and two phase II studies in patients with NASH and cirrhosis are ongoing to evaluate the efficacy.<sup>135</sup> The farnesoid X receptor (FXR) agonist obeticholic acid, improves liver damage and shows beneficial effects on portal hypertension in both NASH and primary biliary cholangitis (PBC).<sup>136</sup>

Vitamin E 800 IU/day, with its antioxidant properties, is used to improve steatosis and inflammation but its effect on fibrosis is currently under investigation.<sup>137</sup> Unfortunately, vitamin E is not recommended to treat diabetic NASH patients with other chronic liver diseases. In addition to vitamin E, silybin, the main compound of the extract from *silybum marianum*, is able to slow fibrosis

progression in different chronic liver diseases through its antioxidant, anti-inflammatory and antifibrotic properties.<sup>138</sup> An Italian randomized placebo-controlled double-blind clinical trial conducted in 70 HCV patients, showed that the combination of pegylated-IFN plus ribavirin with silybin and vitamin E was able both to improve the antiviral response and to reduce serum markers of liver fibrosis through a direct antioxidant effect on the activation of HSCs.<sup>139</sup> Another randomized trial conducted in 99 patients with biopsy proven NASH confirms the antifibrotic effects of silybin but further studies in larger cohorts are required to define its optimum dosage and the correct duration of the treatment.<sup>140</sup> Another antioxidant compound commonly used in traditional medicine is anthocyanin (from plants). In mice models of cholestatic liver damage and bile duct ligation, the decrease in the oxidative stress and lipid peroxidation improve both liver inflammation and fibrosis.<sup>141</sup> Finally, lysyl oxidase monoclonal antibody simtuzumab is currently used in two phase II trials for the treatment of NASH patients with severe fibrosis/cirrhosis but its efficacy is scarce.<sup>142</sup>

## **Conclusions**

The profound understanding of the pathogenetic mechanism of liver fibrosis together with the availability of reliable non-invasive tools for disease assessment allowed significant improvement of management of patients with chronic liver disease. Imaging methods such as transient elastography are currently widely adopted in clinical practice. Serum biomarkers of fibrosis are also well validated in patients with chronic viral hepatitis, despite being less well validated in NAFLD and in other chronic liver diseases. In this context, non-invasive tools permit a reliable detection of cirrhosis which is still a major clinical end-point; indeed, patients with liver cirrhosis should be monitored for the complications related to portal hypertension and screened for HCC development. There is, however, an urgent need to develop probes and contrast agents to non-invasively detect, when it is still reversible, the first signs of fibrosis through multimodal molecular imaging.

To date, the possibility to directly treat liver fibrosis remains an unmet need. Several therapeutic approaches are currently under investigation both at preclinical and clinical level, and have shown promising results. In the near future, the identification of novel potential targets for the development of antifibrotic drugs allowing either profibrogenic pathways inhibition or selective fibrolysis activation could provide powerful tools to arrest and even revert liver fibrosis.

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Table I. *Performance of surrogate serum biomarkers for non-invasive evaluation of significant liver fibrosis and cirrhosis.*

Abbreviations:  $\gamma$ -GT, gamma-glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; BMI, body mass index; HA, hyaluronic acid; IFG, impaired fasting glucose; TIMP-1, tissue inhibitor of metalloproteinase-1.

Figure 1. Mechanisms involved in the pathogenesis of hepatic fibrosis.

In the setting of NAFLD, the impaired lipolysis in the adipose tissue due to insulin resistance leads to an increase flux of FFAs that from the adipose tissue reach the liver. In the liver, FFAs, in part promote the development of steatosis but an excess enhances lipoperoxidation and oxidative stress. Similarly, alcohol consumption and HCV infection contribute to the formation of ROS that, in turn may led to hepatic injury. The intestinal dysbiosis alters the gut microbiome and promotes the release of several PAMPs that can reach the liver and enhance HSCs activation. Genetic background seems to be involved in the onset and progression of hepatic fibrosis, mainly through the *PNPLA3* rs738409 variant. In chronic HBV infection, viral genotype and viral replication are the main factors that contributes to fibrosis progression.

Abbreviations: aHSC, activated hepatic stellate cells; FFAs, free fatty acids; HBV, hepatitis B virus; HCV, hepatitis C virus; HSCs, hepatic stellate cells; IR, insulin resistance; ox-stress, oxidative stress; NAFLD, non-alcoholic fatty liver disease; PAMPs, pathogen associated molecular patterns; PNPLA3, patatin-like phospholipase domain-containing protein 3; qHSC, quiescent hepatic stellate cells; ROS, reactive oxygen species; TG, triglycerides.