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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1689992> since 2019-05-03T11:48:19Z

Published version:

DOI:10.1016/j.mam.2018.09.002

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Invited Review

Liver fibrosis: pathophysiology, pathogenetic targets and clinical issues

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Grant Support: Maurizio Parola was supported by Italian Association for Cancer Research (AIRC; IG 2017 - Id.20361). Massimo Pinzani was supported by NIHR BRCIII University College London.

Conflict of interest: The authors declare no conflict of interest with the content of the present article

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Keywords: hepatic myofibroblasts, hepatic stellate cells, liver fibrosis, liver biopsy, transient elastography.

Abstract

The progression of chronic liver diseases (CLD), irrespective of etiology, involves chronic parenchymal injury, persistent activation of inflammatory response as well as sustained activation of liver fibrogenesis and wound healing response. Liver fibrogenesis, is a dynamic, highly integrated molecular, cellular and tissue process responsible for driving the excess accumulation of extracellular matrix (ECM) components (i.e., liver fibrosis) sustained by an eterogeneous population of hepatic myofibroblasts (MFs). The process of liver fibrogenesis recognizes a number of common and etiology-independent mechanisms and events but it is also significantly influenced by the specific aetiology, as also reflected by peculiar morphological patterns of liver fibrosis development. In this review we will analyse the most relevant established and/or emerging pathophysiological issues underlying CLD progression with a focus on the role of critical hepatic cell populations, mechanisms and signaling pathways involved, as they represent potential therapeutic targets, to finally analyze selected and relevant clinical issues.

Abstract word count: 149 words

1. Liver fibrogenesis and fibrosis in chronic liver diseases: introductory remarks

Chronic liver diseases (CLD) progression, irrespective of the etiology, is characterized by a long-standing history of chronic parenchymal injury, persistent activation of inflammatory response as well as sustained activation of liver fibrogenesis and wound healing response. Liver fibrogenesis, in turn, is a dynamic, highly integrated molecular, cellular and tissue process responsible for driving the progressive excess accumulation of extracellular matrix (ECM) components (i.e., liver fibrosis) and sustained by the activation of hepatic myofibroblasts (MFs), a heterogeneous population of proliferative, migratory and profibrogenic cells that also modulate inflammatory/immune response and angiogenesis (Böttcher and Pinzani, 2017; Higashi et al., 2017; Koyama and Brenner, 2017; Lee et al., 2015; Seki and Schwabe, 2015; Trautwein et al., 2015; Novo et al., 2014).

Although fibrogenesis and fibrosis may represent an attempt to limit the consequences of chronic liver injury within the so-called “chronic wound healing reaction”, they represent key features of the progression of any form of CLD towards liver cirrhosis and hepatic failure. Moreover, liver fibrogenesis and CLD progression are linked to persisting pathological angiogenesis, with angiogenesis contributing to the expansion of tissue fibrosis (Novo et al., 2014; Bocca et al., 2015; Lemoine et al., 2016). The term cirrhosis defines an advanced stage of CLD characterized by an altered structure involving the formation of regenerative nodules of parenchyma surrounded by fibrotic septa as well as significant changes in organ vascular architecture which, in turn, can result in the development of portal hypertension and related complications (variceal bleeding, hepatic encephalopathy, ascites, hepatorenal syndrome, etc.) (Rosselli et al., 2013). Patients undergoing fibrogenic progression are also at significant risk to develop primary liver cancer, in particular hepatocellular carcinoma (HCC) (El Serag, 2011; El Serag, 2012; McGlynn et al., 2015).

In this review the most relevant and/or emerging pathophysiological issues for CLD progression will be summarized, with a first focus on the etiological causes and the global impact of CLDs and an analysis of different morphological patterns of fibrosis. We will next emphasize the role of critical cells, mechanisms and signaling pathways involved in CLD progression (representing key potential therapeutic targets), to finally analyze key relevant clinical issues.

2. Etiology and epidemiology: the global impact of CLDs

CLD represent a major concern for public health worldwide, with more than 800 million people affected and a mortality rate of approx. 2 million deaths per year (Byass, 2014; Marcellin and Kutala, 2018). CLD progression relies mainly on (Arndtz and Hirschfield, 2016; Thrift et al., 2017; Marcellin and Kutala, 2018; Younossi et al., 2018): (i) chronic infection by hepatotropic viruses like hepatitis B virus (HBV, the most common risk factor in Asia) and hepatitis C virus (HCV), both worldwide distributed; (ii) excess alcohol consumption (i.e., alcoholic liver disease or ALD) and (iii) non-alcoholic fatty liver disease (NAFLD), both predominant in western countries; (iv) autoimmune liver diseases, including primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH); (v) hereditary diseases, including Wilson's disease, haemochromatosis and α 1-anti-trypsin deficiency. The worldwide estimated incidence and prevalence of CLDs largely varies depending on the specific etiology, geographic area and likely other factors (sex, race, socioeconomic status) (Marcellin and Kutala, 2018). If we specifically refer to cirrhotic patients, in a large population-based study performed in United States the prevalence of cirrhosis in the general population has been reported to be 0.27%, accounting for more than 600.000 patients (Scaglione et al., 2015). However, this value is likely to be even higher on a worldwide basis since a significant percentage of patients remain asymptomatic and/or is diagnosed only premortem (Marcellin and Kutala, 2018). In addition, decompensated cirrhosis accounts for approximately one million deaths

per year worldwide and 170 000 deaths per year in Europe (Blachier et al., 2013). At present, liver cirrhosis represents the main indication for liver transplantation, with more than 5000 cirrhotic patients being transplanted per year just in Europe (Blachier et al., 2013; Tsochatzis et al., 2014)

Liver cirrhosis is also a major risk to develop HCC that accounts for 75-80% of primary liver malignancies, being the fifth most common solid malignant tumor and the third leading cause of cancer-related death worldwide (approx. 700.000 cases per year, 50.000 per year in Europe) (El Serag, 2011; El Serag, 2012; McGlynn et al., 2015). Of relevance, progressive NAFLD (i.e., non-alcoholic steatohepatitis or NASH), is emerging worldwide as the most rapidly growing indication for liver transplantation in HCC patients, with a significant percentage of cases diagnosed in non-cirrhotic patients (Wong et al., 2014; Younes and Bugianesi, 2018).

3. Liver fibrogenesis: common and etiology-independent issues and mechanisms

3.1. Common and etiology-independent issues in liver fibrogenesis

CLD progression is driven by an interrelated vicious circle of persisting chronic liver injury, chronic inflammation and progressive fibrogenesis and is usually a longstanding process since cirrhosis and its complication develops on average after at least 15-20 years of chronic parenchymal injury. An exception to this long and chronic course is represented by the accelerated fibrosis progression in recurrent hepatitis C (often defined “fibrosing cholestatic hepatitis”) in a significant percentage of liver transplant recipients who develop bridging fibrosis and cirrhosis within the first five years post-transplantation (see references in Berenguer and Schuppan, 2013). Perpetuation of liver injury is also sustained by chronic inflammatory response through a number of damaging mediators, with reactive oxygen species (ROS) and other oxidative stress - related mediators playing a major role (Parola et al., 2008; Novo and Parola, 2008; Novo et al., 2014). Chronic

inflammatory response and the recruitment and activation of either innate or adaptive immune cells is critical in initiating and perpetuating activation of profibrogenic cells into MFs through the release of cytokines, chemokines, ROS and a plethora of other mediators. MFs actively contribute to CLD perpetuation through increased deposition of ECM as well as by releasing cytokines, chemokines and other mediators establishing, together with inflammatory cells, resulting in a “profibrogenic environment” negatively affecting hyperplasia/regeneration in liver parenchyma. (Pellicoro et al., 2014; Seki and Schwabe, 2015; Krenkel and Tacke, 2017). Increased generation of ROS and oxidative stress are involved in almost all conditions of CLD and can be related to the impact of the specific etiology as well as on the activation of resident and recruited cells of innate immunity, with a particular role for NADPH-oxidase activation in different hepatic cell populations following ligand/receptor interactions. ROS and oxidative stress can induce hepatocyte injury and death as well as inhibit parenchymal cell proliferation whilst sustaining directly and indirectly fibrogenesis (Novo and Parola, 2008; Novo et al., 2014). An emerging feature common to different CLD is represented by the role of so-called extracellular vesicles or EVs, (best characterized in progressive NAFLD, see 4.4.2 section), particles of different size that are released by injured and/or apoptotic hepatocytes. EVs, that have been reported to contain signaling proteins, lipids, mRNAs and miRNAs, can act on almost all different cell populations inducing/sustaining inflammation, fibrosis and angiogenesis and have been proposed as putative biomarkers of CLD progression (Kornek and Schuppan, 2012; Povero and Felstein, 2016; Szabo and Momen-Heravi, 2017; Olaizola et al., 2018).

CLD progression is also consequence of excess ECM deposition and of significant changes in the quality and topographic distribution of ECM components paralleled by altered and/or inefficient remodeling and increased expression of tissue inhibitors of metalloproteinases (TIMPs) (Iredale et al., 2013). The replacement of collagen IV in the

space of Disse by fibrillary collagen I and III, following activation of HSC, is a major event known to lead to the capillarization of sinusoids. With the development of fibrotic septa and the ongoing progression towards cirrhosis additional structural changes become evident, including vascular changes due to either hypoxia-dependent or -independent pathological angiogenesis (Novo et al., 2014). The formation of vascular shunts and functional abnormalities due to the endothelial dysfunction generated by an altered ratio between vasodilators and vasoconstrictors (Pinzani et al., 1996; Garcia-Pagan et al., 2012) will result in the genesis of portal hypertension and related clinical complications. There is evidence derived from animal models and clinical studies suggesting that liver tissue fibrosis can be halted and even reversed after the withdrawal of the underlying cause of disease. This is associated with a significant and progressive decrease of the number of MFs either because of apoptosis, induction of senescence and killing of senescent HSC by NK cells or phenotypic reversion to a more quiescent phenotype. This regression is associated with a reduction of collagen as well as of TIMP-1 expression and an increase in hepatic collagenase and elastase activity resulting in ECM degradation and remodeling. (Pellicoro et al., 2014; Lee et al., 2015; Ramachandran et al., 2015; Campana and Iredale, 2017).

3.2 Role of innate and adaptive immune cells and other cells in CLD progression

3.2.1 Kupffer cells and monocyte-derived macrophages in CLD

Hepatic macrophages, essential to maintain tissue homeostasis and ensure rapid responses to hepatic injury, include Kupffer cells (KC, liver resident and self-sustaining macrophages) and monocyte-derived macrophages (MoMF) that accumulate in the injured liver. These cells, on the basis of specific signals, can adapt their polarization and can either promote restoration of tissue integrity following acute injury or, in case of chronic injury, contribute to CLD progression. Studies performed in mice have outlined an

extremely complex scenario that is currently superseding the traditional and schematic distinction between M1 and M2 polarization (Krenkel and Tacke, 2017; Tacke, 2017).

In case of liver injury, KC (CD11b⁺, F4/80⁺⁺, CD68⁺, CXCR1⁻) are activated by various damage-associated molecular patterns (DAMPs, like free DNA, ATP, FFA, high-mobility group box 1) and pathogen-associated molecular patterns (PAMPs, like LPS or viral DNA) interacting with Toll-like receptors (TLRs) or P2X7 receptor. This results in inflammasome assembly and activation in KC and in the release of interleukin-1 β (IL-1 β), IL-18 and various other pro-inflammatory cytokines and chemokines (in particular CC-chemokine ligand 2 or CCL2), leading to recruitment of circulating leukocytes (monocytes and neutrophils), modulation of T cells, and increased expression of vascular adhesion molecules on sinusoidal endothelial cells (SEC). In experimental murine models CCL2 and other chemokines promote the recruitment of CCR2⁺/Ly-6C^{hi} monocytes into injured liver, where they develop into inflammatory, angiogenic and fibrogenic Ly-6C⁺ macrophages. These Ly-6C⁺ macrophages in CLD can activate HSC or other precursor cells to become collagen-producing MFs by releasing several mediators, with TGF β 1 and PDGF being the most relevant ones. Moreover, fibrogenic Ly-6C⁺ macrophages can enhance MFs survival through stimulation of nuclear factor kappa B (NF- κ B) (Pradere et al., 2013) and recruit MFs through chemokines like CCL2 (Pellicoro et al., 2014; Tacke, 2017). In humans, CD14⁺CD16⁺ MoMF were found to have stellate cell-activating capacities. In case of cessation of injury, as in resolution of acute liver injury or following withdrawal of underlying etiological cause in progressive CLD, the decrease in DAMPS and phagocytosis of cell debris (Ramachandran et al., 2012; Wang et al., 2014; Tacke, 2017), with a possible contribution of activation of autophagy (particularly in NASH) (Lodder et al., 2015) and of fractalkine or CX3CL1 (Karlmark et al., 2010), can favor the switch of Ly-6C⁺ macrophages into Ly-6C^{low} restorative macrophages (positive for CX₃CR1, CD206 and Arginase 1 and 2). Restorative macrophages release anti-inflammatory cytokines (IL-

10, IL-1Ra), regenerative growth factors (HGF, VEGF) and matrix degrading metalloproteinase (MMP) expression (MMP9, MMP12 and MMP13), that promote resolution from injury.

In addition to this extremely simplified general scheme of events, both HBV and HCV can activate human macrophages that respond to HBV by expressing inflammatory cytokines and stimulating NK cells (Tu et al., 2008; Boltjes et al., 2015) or to HCV and HCV-proteins by a TLR2-dependent inflammasome activation (Chang et al., 2007; Hosomura et al., 2011; Srivastava et al., 2013). Interestingly, while the pro-inflammatory profile of cytokines can promote hepatitis and apoptosis of HCV infected hepatocytes, TLR3-mediated antiviral activities (interferon release, TRAIL expression) are deactivated (Tu et al., 2010). Even more relevant, during chronic HBV and HCV infection human macrophages also release immunomodulatory mediators (IL-10, TGF β 1, galectin-9, PD-L1 and PD-L2) that, with the time, contribute to suppress antiviral T cell response (Ju and Tacke, 2016).

In progressive NAFLD hepatic macrophages display an inflammatory phenotype (positive for Ly-6C, iNOS, TNF, IL-1 β and CCL2) that depends also on the excess of lipids and FFA (Jindal et al., 2015), signals from surrounding fat-laden hepatocytes like EVs (Hirsova et al., 2016; Cannito et al., 2017a) or histidine rich glycoprotein (Bartneck et al., 2016; Morello et al., 2018). Indeed accumulation of inflammatory macrophages is a hallmark of human NAFLD progression (Wan et al., 2014; Gadd et al., 2014) that these cells may sustain by influencing steatosis, stimulating angiogenesis and sustaining fibrosis.

In ALD patients macrophages are particularly enriched in the portal tracts and have a central role in promoting inflammation associated with ethanol-induced injury, particularly in severe alcoholic hepatitis (Ju and Mandrekar, 2015; Suraweera et al., 2015) where increased gut permeability and high portal levels of endotoxins contribute to accumulation and exacerbated activation of Ly-6C^{hi} murine macrophages and the release of TNF, ROS and CCL2 (Petrasek et al., 2012; Wang et al., 2014; Ju and Tacke, 2016).

In cholestatic conditions, macrophage functions are altered and bile acids and bile acid composition can significantly affect responses of MoMF (Calmus and Poupon, 2014). For example, chenodeoxycholic acid (CDCA), leads to NLRP3 inflammasome activation and IL-1 β secretion in murine macrophages (Gong et al., 2016). However, this is not the case for KC that express the bile acid receptor TGR5 (G-protein-coupled bile acid receptor 1), allowing these cells to sense bile acids mainly for anti-inflammatory responses (Keitel et al., 2008; Duwaerts et al., 2013) and to block inflammasome (Guo et al., 2016).

3.2.2 Role of other innate or adaptive immune cells in progressive CLD

Whether other innate immune cells are concerned, neutrophils do not apparently sustain fibrogenesis but rather contribute to collagen degradation during resolution of injury by releasing MMPs (Saito et al., 2003; Harty et al., 2010). A pro-fibrogenic role for liver dendritic cells (DC), which are known to modulate hepatic immune responses (Plitas et al., 2008; Pellicoro et al., 2014), has been proposed in a preclinical study in which efficient DC depletion prevented the development of fibrosis (Connolly et al., 2009).

Concerning the cells of adaptive immunity, almost all functional lineage of cells derived from naïve CD4⁺ lymphocytes can have a role in modulating liver fibrosis. T_H2 cytokine response, mediated by IL-4, IL-5 and IL-13, can operate as pro-fibrogenic in progressive CLD (Pellicoro et al., 2014), with IL-13 being the most relevant mediator at least in experimental model of liver fibrosis (Chiamonte et al., 1999; Shimamura et al., 2008; Weng et al., 2009). IL-13, through binding to IL-13R α 1, can elicit profibrogenic and proliferative responses of HSC or other precursor cells in a TGF β 1-independent way, possibly by inducing connective tissue growth factor (Liu et al., 2011; Kaviratne et al.; 2004). However, in mice lacking the IL-13R α 2, a decoy receptor also expressed on MFs, liver fibrosis due to *Schistosoma mansoni* is accelerated (Mentink-Kane et al., 2011), leading to the suggestion that IL-13R α 2 may operate as a regulator of both T_H17 mediated inflammation and T_H2-mediated fibrosis (Mentink-Kane and Wynn; 2004). T_H17

lymphocytes, a subset of T helper cells releasing the pro-inflammatory IL-17A, may also behave as pro-fibrogenic in HBV- and HCV-related CLD (Wang et al., 2011; Paquissi, 2017) and other conditions, possibly in relation of NLRP3 inflammasome activation (Wree et al., 2017) or, as proposed for cholestatic diseases, following release of IL-17 by intrahepatic $\gamma\delta$ T-cell-receptor positive cells under conditions of altered intestinal microbiota (Tedesco et al., 2018).

TH1 responses, mainly through interferon- γ (IFN- γ) released by TH1 lymphocytes, NK or NK-T cells are usually antifibrotic through different mechanisms (Svegliati-Baroni et al., 1996; Jeong et al., 2008). In particular, NK cells can operate as cells able to specifically kill senescent HSC and, through their release of IFN γ , reinforced by IL-15, to induce cell cycle arrest and apoptosis in HSC (Radaeva et al., 2006; Jiao et al., 2016). However, CD4+ T lymphocytes, by interacting with either NK cells and activated HSC, can suppress NK cells favoring HSC survival (Langhans et al., 2015). Although NK-T have been reported to kill activated HSC, it should be noted that a subset of these cells can release IL-4, IL-13 and Hedgehog ligands as well as to promote HSC activation and liver fibrosis (Gao and Radaeva, 2013).

More complex is the role of CD4+/CD25+/Foxp3+ T regulatory (Treg) cells that are significantly induced in CLD and reported to suppress HCV-related liver fibrosis (Claassen et al., 2010) but to promote fibrogenesis in other organs (Cannito et al., 2017b).

Finally, there is limited experimental evidence that B lymphocytes may also contribute to liver fibrogenesis (Novobrantseva et al., 2005; Thapa et al., 2015).

3.2.3 Role of hepatic progenitor cells and activated cholangiocytes

Under condition of chronic liver injury, persistent inflammation and oxidative stress, hepatocyte proliferation and hepatocellular differentiation from adult liver stem cells are inhibited, with transient amplifying hepatic progenitor cells (HPCs) being forced to mainly differentiate into activated and stress-resistant cholangiocyte-like - or reactive ductular

cells (RDS) in an overall scenario defined “ductular reaction”. These pro-inflammatory and profibrogenic cells, actively contributing to progressive CLD of any etiology at advanced stage (Lowe et al., 1999; Roskams et al., 2003; Clouston et al., 2005), have a particularly prominent role in progressive NAFLD and chronic diseases involving the biliary tract (Richardsson et al., 2007; Penz-Österreicher et al., 2011; Gadd et al., 2014). In particular, RDS (and possibly HPCs), can release several pro-fibrogenic mediators (TGF β 1, TGF β 2, PDGF-BB, CTGF, sonic Hedgehog, ET-1) that allow intense cross-talk with surrounding portal fibroblasts or HSC, eliciting and sustaining the activity of MFs (Milani et al., 1991; Pinzani et al., 1996; Omenetti et al., 2008). These MFs, in turn, can release survival factors and other mediators (HGF, IL-6, bFGF, etc.) that contribute to perpetuate activation of these cholangiocytes and then, in a vicious circle, fibrogenesis (Schuppan et al., 2018). Moreover, in experimental NASH hepatocytes respond to Jag1 from HSC or MFs by eliciting a Notch-dependent signaling able to sustain ductular reaction (Morell et al., 2017). Finally, RDS can release pro-inflammatory mediators which may in part explain the typical portal/periportal inflammation in cholangiopathies intimately associated with biliary-like fibrosis. At present more convincing data are related to a murine model of congenital hepatic fibrosis (CHF, Pkhd1 mice), in which cholangiocytes, defective for the gene encoding fibrocystin (FPC), in the early phase of the disease recruit inflammatory cells by secreting chemokines like CXCL1, CXCL10, and CXCL12 (Fabris et al., 2017).

3.3 Origin and role of hepatic myofibroblasts

Hepatic MFs represent a heterogeneous population of highly proliferative and contractile α -SMA-positive cells that can originate from different mesenchymal precursor cells through a process of activation/transdifferentiation (Friedman, 2008; Novo et al., 2014; Lee et al., 2015; Seki and Schwabe, 2015; Trautwein et al., 2015; Wells and Schwabe, 2015; Tsuchida and Friedman, 2017). Persistently activated pro-fibrogenic MFs act as a unique

crossroad cell type able to integrate incoming paracrine/autocrine signals from the “profibrogenic environment” (ROS, growth factors, cytokines, chemokines, adipokines, proangiogenic mediators, etc.) and released by both hepatic (hepatocytes, KC, SEC, cholangiocytes, HPCs, resident lymphocytes) and extrahepatic (infiltrating innate and adaptive immune cells and other bone marrow-derived cells) populations involved in CLD progression.

3.3.1 Hepatic stellate cells as the major source of liver MFs

Hepatic stellate cells (HSC) in the normal liver reside in the sub-endothelial space of Disse and establish intimate contacts with surrounding hepatocytes, SEC, other HSC and nerve endings through their cytoplasmic processes. HSC are physiologically responsible for synthesis and remodeling of ECM in the space of Disse, for storage and metabolism of vitamin A and retinoids, being also able to operate as liver specific pericytes. Under conditions of chronic liver injury, HSC become activated through a process of activation and trans-differentiation into MF-like cells (HSC/MFs or MFs derived from HSC), a process elicited and sustained by the “profibrogenic environment” and signals from involved cell populations mentioned before. HSC/MFs are cells exhibiting all the peculiar characters of hepatic MFs (see later in 3.3.6) (Friedman, 2008; Tsuchida and Friedman, 2017). HSC/MFs abundantly synthesize ECM components, particularly fibrillary collagens and at least initially in the space of Disse (i.e., leading to sinusoidal capillarization), loose cytoplasmic droplets containing vitamin A and retinoids, and release endothelin 1, a potent vasoconstrictor that promotes proliferation, fibrogenesis and contraction and has been linked to portal hypertension in cirrhotics (Friedman, 2008; Igashi et al., 2017). Experimental and clinical data indicate that activated/transdifferentiated HSC represent the major source of hepatic MFs, whatever the etiology (Friedman, 2008; Tsuchida and Friedman, 2017). According to elegant fate tracing studies HSC/MFs account for 82-96% of all MFs in most murine models of CLD (Mederacke et al., 2013), with the possible

exception of the bile-duct ligation (BDL) model of biliary fibrosis in which a higher contribution of portal MFs has been proposed (Iwaisako et al., 2014; Wells and Schwabe, 2015). In addition to α -SMA, HSC/MFs are positive for additional specific markers, including glial fibrillary acidic protein (GFAP), nerve growth factor receptor (p75), platelet-derived growth factor (PDGF) receptor β (PDGFR β), lecithin-retinol acyltransferase (LRAT), integrin α v β 3, vimentin, desmin, mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF-IIR) and cytoglobin (Friedman, 2008; Kawada, 2015; Higashi et al., 2017). HSC have been also analyzed by employing genome wide transcriptome profiling that has outlined 122 HSC-specific genes and 194 HSC-specific gene signatures associated with poor patient prognosis in CLD and HCC development (Eggert et al., 2016; Zhang et al., 2016).

3.3.2 MFs from portal fibroblasts

Hepatic MFs can originate from portal fibroblasts, a population of resident fibroblasts located in the mesenchyme of portal areas which surrounds bile ducts proposed to be mainly involved in conditions of biliary fibrosis. Portal MFs exhibit a peculiar marker profile by expressing, in addition to α SMA, fibulin 2, elastin, IL-6, cofilin 1 and the ecto-ATPase nucleoside triphosphate diphosphohydrolase-2 (NTPD2) (Dranoff and Wells, 2010; Wells and Schwabe, 2015). At present, two fate tracing studies have provided conflicting results on the relative contribution of portal MFs in experimental conditions of biliary fibrosis, with one study suggesting that as much as 70% of MFs were derived from portal fibroblasts (Iwaisako et al., 2014) whilst another study suggested a more limited contribution (Mederacke et al., 2013). Along these lines, portal fibroblasts may represent the earliest cell population activated following injury to cholangiocytes of the biliary epithelium (Kinnman and Housset, 2002; Lemoine et al., 2013).

3.3.3 MFs from bone marrow – derived precursors

A limited percentage of hepatic MFs may originate from bone marrow-derived precursor cells recruited in a chronically injured liver, as initially proposed by a human study showing that in women that developed chronic HCV-related CLD after receiving a bone marrow transplant from male donors, a significant number of hepatic MFs were positive for Y chromosome (Forbes et al., 2004). These findings were confirmed by other experimental studies that suggested that these MFs may originate from either mesenchymal stem cells (Russo et al., 2006; Valfrè di Bonzo et al., 2008) or from α -SMA negative precursor cells defined as fibrocytes (Kisseleva et al., 2006).

3.3.4 MFs from epithelial cells following EMT

As for other conditions of tissue and organ fibrosis liver MFs may originate from hepatocytes or cholangiocytes through a process of epithelial-to-mesenchymal transition (EMT) (Cannito et al., 2017b). However, the involvement of EMT in liver fibrogenesis is at present highly debated and controversial, with a prevailing view suggesting that this profibrogenic mechanism in progressive CLD should be considered of minor relevance (Forbes and Parola, 2011; Xie and Diehl, 2013; Munker et al., 2017) as indicated by several negative fate tracing murine studies (Taura et al., 2010; Scholten et al., 2010; Chu et al., 2011; Österreicher et al., 2011; Mederacke et al., 2013; Iwaisako et al., 2014). A possible exception may be represented by the reported Hedgehog pathway-mediated transition of cholangiocytes into pro-fibrogenic MFs under conditions of NAFLD/NASH and (see more details later in 4.4.2 section) related to ROS, HPC and ductular reaction (Syn et al., 2009).

3.3.5 MFs from Mesothelial cells

In mouse embryos liver mesothelial cells (MCs) of the Glisson's capsule were found to migrate within the liver to give rise to HSC, fibroblasts, and vascular smooth muscle cells

(Asahina et al., 2011). Glycoprotein M6a (GPM6A)-positive MCs isolated from adult liver, lose their phenotype to acquire a mesenchymal-like phenotype in a TGF- β -dependent manner. Moreover, using conditional cell lineage tracing in *Wt1^{CreERT2}* mice, MCs were shown to give rise to both HSCs and MFs through a process of mesothelial–mesenchymal transition (MMT) under conditions of experimental chronic injury (Li et al., 2013). The relative contribution of MCs as a source of MFs, however, should be considered as minor or negligible.

3.3.6 Major phenotypic responses of hepatic MFs

Our present knowledge concerning phenotypic profibrogenic responses operated by hepatic MFs largely comes from studies on HSC and HSC/MFs performed either *in vivo* or *in vitro* by taking advantage of primary culture or immortalized cell lines of human or rodent HSC (Higashi et al., 2017) as well as of immortalized rat portal MFs (Fausther et al., 2015). Here we will briefly recapitulate MF-operated major phenotypic responses and their role (Friedman, 2008; Tsuchida and Friedman, 2017).

(1) *Synthesis and remodeling of ECM.* Increased synthesis of ECM components is a hallmark of MFs in progressive CLD, with TGF- β 1 (released by either activated macrophages or HSC/MFs) being the most potent cytokine inducing the production of fibrillary collagens (mainly Type I and III), α -SMA, laminin and fibronectin. This feature is associated to a dysregulation of the expression of genes involved in ECM remodeling, resulting in increased expression of TIMPs and inefficient removal of excess fibrillary collagen by metalloproteases (MMPs). Several other mediators have been proposed to be involved, including: i) ROS released by injured hepatocytes or overproduced as a consequence of activation of NADPH-oxidase isoforms, associated to the interaction of growth factors, cytokines and other active peptides with their cognate receptors; ii) aldehydic products like acetaldehyde (during ethanol metabolism) or 4-hydroxy-nonanal,

the most relevant aldehydic end-product of lipid peroxidation; iii) several growth factors, ligand peptides and signaling pathways.

(2) *Proliferation and survival.* MFs are highly proliferating cells as a result of increased availability of mitogenic growth factors released by surrounding cells in the profibrogenic environment and increased expression of related receptors by MFs. MFs mainly proliferate in response to platelet-derived growth factor (PDGF) or to other mitogens, including transforming growth factor (TGF)- α , epidermal growth factor (EGF), thrombin, keratinocyte growth factor, connective tissue growth factor (CTGF), bFGF and the adipokine leptin. These mitogenic signals (plus TGF β 1) are believed to be responsible for the increased survival and resistance to apoptotic stimuli of MFs that in their state of persistent activation.

(3) *Pro-inflammatory role.* MFs, in addition to express receptor for several cytokines and other inflammatory mediators, can contribute to perpetuate inflammatory response and regulating and/or modulating interactions with cells of innate and adaptive immunity by synthesizing and releasing critical pro-inflammatory mediators, in particular the chemokines CCL2 and CCL21 as well as IL-1 β following activation of NLRP3 inflammasome.

(4) *Migration.* MFs acquire the ability to migrate in a scenario of progressive CLD in response to several peptide chemoattractants released by surrounding liver cell populations or by MFs themselves and/or trapped in the ECM, including PDGF, CCL2, Angiotensin II, VEGF-A and Angiopoietin 1. MFs migration by these chemoattractants is mediated through an increase of intracellular ROS levels, leading to activation of ERK1/2 and JNK1/2 signaling pathways, that depends on ligand/receptor-related NADPH oxidase activation or ROS released by mitochondria under hypoxic conditions (Novo et al., 2011; Novo et al., 2012).

(5) *Pro-angiogenic role.* HSC/MFs have been reported to synthesize and release proangiogenic mediators like VEGFA, Angiopoietin-1 or -2, PDGF-BB and hedgehog

ligands. HSC/MFs and MFs can express related cognate receptors, then also representing a cellular target for these mediators. Since hypoxia-dependent angiogenesis usually precedes or parallels fibrosis, it has been proposed that it may drive fibrogenesis and the formation of fibrotic septa (Novo et al., 2014; Bocca et al., 2015; Lemoine et al., 2016).

4. Established and emerging etiology-related issues

The specific etiology has a relevant impact in modulating fibrogenic CLD progression leading to distinct morphological patterns of fibrosis development that also depend on the origin of prevailing profibrogenic cell types and mechanisms involved (Pinzani and Rombouts, 2004; Böttcher and Pinzani, 2017). The different and etiology-dependent patterns of liver fibrosis depend on some critical issues: 1) the effective location of liver injury, according to the specific etiology; 2) the actual concentration of pro-fibrogenic factors in the microenvironment; 3) the prevailing pro-fibrogenic mechanism(s) involved; 4) the specific cellular origin of MFs.

4.4.1 Etiology-related issues in HBV- and HCV- mediated progressive CLD

In HBV- and HCV-related CLD the prevailing pattern of fibrosis evolution is referred to as post-necrotic or bridging fibrosis, being characterized by increased deposition of ECM components under the form of portal-central (vein) fibrotic septa driven or resulting by portal–central bridging necrosis. This pattern is also characterized by the presence of the so-called interface hepatitis, the formation of portal-portal septa and of blind septa into the injured liver parenchyma. As a consequence, an early and rapid derangement of the vascular connections within the portal system can occur, including formation of neo-vessels and porto-central shunting, resulting in an earlier development of portal hypertension (Pinzani and Rombouts, 2004; Böttcher and Pinzani, 2017).

The prevalent pro-fibrogenic mechanism in HBV- or HCV-related CLD progression is represented by chronic activation of wound healing, with ROS and oxidative stress also offering a relevant contribution (Pinzani and Rombouts, 2004; Parola et al., 2008; Novo and Parola, 2008; Böttcher and Pinzani, 2017). Data from human studies support the concept that here hepatic MFs originate mainly from activated HSC and from activated portal fibroblasts, with a minor contribution of MFs from bone-marrow derived cells.

Some critical considerations should be underlined in relation to HBV or HCV-dependent fibrosis. A first notion is that both HBV and HCV are non-cytopathic viruses for hepatocytes and that liver parenchymal injury and hepatocyte death should be attributed to the host's immune response in an attempt to clear viruses (Guidotti and Chisari, 2006). However, the overall response from virus-specific CD4+ and CD8+ T lymphocytes with the time becomes inefficient or exhausted and then unable to completely clear HBV or HCV from the liver. This will result in chronic infection, cycles of low-level cell injury and persistent inflammatory response, in which the secondary recruitment of non-antigen specific mononuclear cells (with NK cells playing a major cytotoxic and/or regulatory role) is believed to be critical in sustaining parenchymal damage, inflammatory response and fibrogenic CLD progression (Protzer et al., 2012; Behermann, 2013).

Another peculiar issue is represented by a direct role played by specific HCV proteins like core and NS3/NS5 proteins. Although HCV does not infect HSC, these proteins can stimulate activated human HSC in a ROS- and redox-dependent manner, leading to up-regulation of pro-fibrogenic and pro-inflammatory responses (Bataller et al., 2004). In addition, HCV proteins can induce increased intracellular generation of ROS in hepatocytes, then potentially contributing to oxidative-stress mediated hepatocyte injury and, as for other etiologies, activation/perpetuation of phenotypic responses of HSC (Choi and Hou, 2006; Novo and Parola, 2008; Novo et al., 2011; Novo et al., 2014).

4.4.2 Etiology-related issues in progressive NAFLD/NASH

Progressive NAFLD is emerging as a leading cause of end-stage liver disease worldwide, with a 23% estimated prevalence in European general population, occurring in the context of the metabolic syndrome and reaching 70-90% prevalence among obese and type II diabetic patients (Rosselli et al., 2014; Younossi et al., 2018). About 20-30% of NAFLD patients develop non-alcoholic steatohepatitis (NASH), characterized by parenchymal injury, lobular/portal inflammation and perisinusoidal fibrosis, that can progress to cirrhosis and HCC (Yeh and Brunt, 2014; Satapathy and Sanyal, 2015). Progressive NAFLD, similarly to ALD, is characterized by a peculiar type of fibrosis development in which excess deposition of ECM components is initially mainly observed around the sinusoids (peri-sinusoidal fibrosis, responsible for sinusoidal capillarization) and around groups of hepatocytes (peri-cellular fibrosis or “chicken wire” pattern) (Pinzani and Rombouts, 2004). Accordingly, fibrogenic progression of NAFLD relies mainly on the involvement/activation of HSC as a major source of hepatic MFs (Friedman, 2008; Lee et al., 2015; Seki and Schwabe, 2015; Trautwein et al., 2015; Tsuchida and Friedman, 2017), although EMT of cholangiocytes may occur (Syn et al., 2009).

According to epidemiological studies, NASH develops only in a fraction of NAFLD patients while the majority presents only steatosis. Chronic hepatic injury, inflammatory response and fibrogenesis in progressive NAFLD are highly affected/modulated by “multiple parallel hits” involving several mechanisms and pathophysiological issues as well as the interaction of the liver with adipose tissue and intestine in terms of release and action of growth factors, cytokines, adipokines and other mediators (Tilg and Moschen, 2010; Moschen et al., 2013; Tilg et al., 2017; Schuppan et al., 2018; Marra and Svegliati Baroni, 2018).

Hepatic steatosis develops as a consequence of increased delivery of free fatty acids (FFA) from insulin resistant adipose tissue, intrahepatic de novo lipogenesis and excess

dietary lipids (Donnelly et al., 2005; Marra and Svegliati Baroni, 2018). However, parenchymal injury and then progressive NAFLD are mostly related to lipotoxicity and other pathophysiological issues, with the nutrient/caloric overload (i.e., not matched by adequate energy expenditure) and the role of dysfunctional adipose tissue having a central role (Tilg and Moschen, 2010; Moschen et al., 2013; Tilg et al., 2017; Schuppan et al., 2018; Marra and Svegliati-Baroni, 2018).

Weight gain in overweight/obese patients results in expanded and hypoxic adipose tissue and adipocyte apoptosis, leading to CCL2-dependent recruitment of M1 polarized macrophages. These macrophages, by secreting pro-inflammatory cytokines, contribute to insulin resistance and to the related lipolysis and increased hepatic delivery of free fatty acids (FFA). The expanded and inflamed adipose tissue also causes an imbalance in adipokine secretion resulting to an increase in leptin and a decrease in adiponectin circulating levels, that have an additional role in progressive NAFLD (Marra and Lotersztajn, 2013; Schuppan et al., 2018; Marra and Svegliati-Baroni, 2018).

Hepatocyte injury does not depend on fat accumulation, since the block of triglyceride synthesis prevents steatosis but is associated with increased oxidative stress, inflammation and fibrosis (Yamaguchi et al., 2007; Marra and Lotersztajn, 2013). Rather, in a scenario of excess availability of FFA, hepatocyte injury depends on lipotoxicity exerted in particular by saturated FFA like palmitate and stearate, lysophosphatidylcholine, ceramides, free cholesterol as well as short chain fatty acids. These toxic lipids induce cell death and particularly lipo-apoptosis acting through multiple mechanisms, including activation of signaling cascades (JNK, Sirtuin 1 and 3, lysosomal pathway and cathepsin B) and death receptors, ER stress, mitochondrial damage and functional dysregulation as well as oxidative stress (Marra and Lotersztajn, 2013; Marra and Svegliati-Baroni, 2018). Increased generation of ROS in fat-laden hepatocytes is related to lipotoxicity, mitochondrial overflow of FFA and related dysfunction but also to increased up-regulation

of cytochrome P450 2E1. In addition, lipotoxicity is believed to cause the release of EVs from fat-laden damaged/apoptotic hepatocytes: these EVs can exert pro-angiogenic and pro-inflammatory effects, up-regulate NLRP3 inflammasome in either hepatocytes and macrophages and to directly act (through their miRNAs content) on HSC (Povero et al., 2013; Povero et al., 2015; Hirsova et al.; 2016; Cannito et al., 2017a).

Defective autophagy has also been reported to be strictly related to steatosis and lipotoxicity, possibly through a saturated FFA-dependent and Sirtuin 3-mediated mechanism (Li et al., 2017). Intriguingly, although autophagy is protective against steatosis in hepatocytes, activation of autophagy can result in the release of lipids able to promote fibrogenesis by activated HSC in mice and humans (Hernandez-Gea et al., 2012).

Another critical issue in progressive NAFLD is represented by the role of changes in intestinal microbiome, gut dysbiosis (resulting also in altered gut barrier function) and altered gut-liver axis. Relevant features are the following: a) bacterial products, through binding to specific Toll-like receptors (TLRs) on hepatic cells, can trigger pro-inflammatory pathways concurring to NASH but also to adipose tissue inflammation; in addition, bacterial products can also indirectly affect glucose metabolism by regulating the release of incretins like glucagon-like peptide-1 (GLP-1); b) synthesis of SCFAs from gut microbes that can act as lipid precursors in the liver (up-regulating lipogenesis and gluconeogenesis), as toxic lipids as well as ligands for a number of G-protein coupled receptors mediating several effects; c) suppression of the synthesis of angiopoietin-related protein 4 or ANGLPTL4, a lipoprotein-lipase inhibitor, resulting in increased fat deposition in both hepatocytes and adipocytes (Marra and Svegliati-Baroni, 2018). An additional issue in the scenario of dysregulated gut liver axis is represented by the knowledge that changes in the composition of gut microbiota may affect levels of bile acids (BAs, which are metabolized by gut bacteria) and then pathways that are regulated by BAs acting as ligands of farnesoid-X receptor (FXR). FXR are nuclear receptors expressed both by

hepatocytes and enterocyte whose activation in hepatocytes can affect different pathways involved in fatty acid metabolism or, through release of FGF15/19 by enterocytes due to local FXR activation, influence lipogenesis and gluconeogenesis through binding in the liver to FGFR4 complexed with β -Klotho (Marra and Svegliati-Baroni, 2018).

NASH progression, which is characterized by a typical and substantial inter-patient variability, is also significantly associated to- and affected by a number of genetic determinants and epigenetic factors (Anstee et al., 2016; Eslam et al., 2018). In recent years at least four well defined genetic variants have been associated with NAFLD susceptibility and development. These genes encode for proteins involved in the regulation of hepatic lipid metabolism, including PNPLA3 (patatin-like phospholipase-domain containing proteins), TMS6F2 (transmembrane 6 superfamily member 2), MBOAT7 (membrane-bound O-acytransferase domain containing 7) and glucokinase regulator (GCKR) (Eslam et al., 2018). The best characterized and validated are genetic polymorphisms in PNPLA3 (Romeo et al., 2008) and TMS6F2 (Kozlitina et al., 2014; Holmen et al., 2014). In particular, a single-nucleotide polymorphism in PNPLA3 involving isoleucine to methionine substitution at position 148 (I148M) is associated to advanced liver fibrosis and confers a 10 fold increased risk to develop HCC (Liu et al., 2014a; Singal et al., 2014; Trepò et al., 2016). The TMS6F2 polymorphism predicts NASH progression and is also associated with an improved cardiovascular disease outcome (Liu et al., 2014b; Anstee et al., 2016). The list of genetic variants associated with progressive NAFLD is continuously growing, as up-dated recently (Eslam et al., 2018), and an additional role in progressive NAFLD is likely to be paralleled and possibly overwhelmed in the future by the role of epigenetic factors, as the first emerging data (mainly related to DNA methylation and chromatin remodeling or to the action of non-coding RNAs) seem to suggest (Anstee et al., 2016; Eslam et al., 2018).

4.4.3 Etiology-related issues in progressive alcoholic liver disease (ALD)

ALD, similarly to NAFLD, is characterized by a spectrum of histopathological lesions that range from simple steatosis to alcoholic hepatitis or alcoholic steatohepatitis (ASH), fibrosis and cirrhosis, with ALD patients also experiencing a significant risk to develop HCC. ASH, in particular, is believed to be critical in driving a fast progression of ALD towards cirrhosis and to increase the risk of decompensation, liver failure and poor outcome (Louvet and Mathurin, 2015). ALD shares with NAFLD the pattern of perisinusoidal/pericellular fibrosis development and a major role proposed for HSC/MFs (Pinzani and Rombouts, 2004; Böttcher and Pinzani, 2017). Interestingly, although cessation of alcohol consumption can favor recovery from fatty liver, alcoholic hepatitis and fibrosis and also improve outcome for cirrhotic, no regression of cirrhosis in ALD patients has been documented (Louvet and Mathurin, 2015).

Chronic hepatocellular injury and death is intimately related to oxidative ethanol metabolism by alcohol dehydrogenase and, particularly, the ethanol-inducible CYP2E1 cytochrome P450 isoform, leading to acetaldehyde, increased ROS generation and oxidative stress-mediated injury (exacerbated by iron and hypoxia), mainly through lipid peroxidation affecting integrity of mitochondria and ER biomembranes (Lieber, 2004; Gao and Bataller, 2011; Cederbaum, 2012). In addition, as for ROS and other oxidative stress mediators (Novo and Parola, 2008), acetaldehyde can directly activate HSC/MFs and to stimulate expression of collagen type I (Mello et al., 2008). ROS are believed to be major responsible for induction of ER stress and, together with acetaldehyde, also of alcohol-induced steatosis, the latter recognizing AMPK downregulation and related SREBP1c stimulation and PPAR- α expression inhibition as critical determinants (Louvet and Mathurin, 2015). DAMPs, released following necrotic cell death, trigger macrophage and neutrophil activation, with senescence (via NK cells) and autophagy being major regulators of liver inflammation (Luedde et al., 2014; Dolganiuc et al., 2012).

A major role in ALD pathogenesis is also attributed to bacterial translocation due to increased gut permeability with increased circulating levels of LPS correlating with the severity of hepatic injury (Rao, 2009). LPS can activate pro-fibrogenic HSC/MFs either directly through activation of TLR4 expressed by these cells (Brun et al., 2005; Seki et al., 2007) or indirectly by stimulating KC and MoMF (Purohit and Brenner, 2006).

Adaptive immunity might also contribute to ALD progression, with chronic alcohol consumption leading to increased levels of antibodies directed against lipid peroxidation products. These antibodies can activate an adaptive immune response, likely by stimulating splenic T cells and NKT cells to develop Fas and/or TNFR1 receptor mediated cytotoxicity towards hepatocytes (Minagawa et al, 2004; Szabo and Mandrekar, 2009). Finally, ethanol can suppress the anti-fibrotic and pro-resolution function of NK cells which is believed to operate through IFN- γ secretion and the related killing of activated HSC (Jeong et al., 2008).

4.4.4 Etiology-related issues in chronic diseases of biliary tract

Chronic diseases of the biliary tract or cholangiopathies include a group of diseases due to immune mediated etiology (i.e., autoimmune diseases like primary biliary cirrhosis or PBC, and primary sclerosing cholangitis or PSC), presenting as congenital conditions (congenital hepatic fibrosis or CHF, biliary atresia and Alagille syndrome) or due to other inflammatory, toxic, ischemic or infectious causes (Fabris et al., 2017). Whatever the etiology, cholangiopathies have in common chronic damage to cholangiocytes of the biliary tree and share similar pathophysiological mechanisms, including proliferation, apoptosis, cholestasis, inflammation, fibrogenesis, and eventually carcinogenesis (Fabris et al., 2017). Persistent injury to biliary epithelium triggers a pathological reparative reaction associated with an excessive deposition of ECM components in areas surrounding injured bile ducts, usually referred to as the biliary fibrosis pattern. Biliary fibrosis, in the scenario

of ductular reaction, is typically characterized by co-proliferation of RDC and MFs in portal areas and at the parenchyma/portal interface, with MFs originating mainly from portal fibroblasts and HSC (Pinzani and Rombouts, 2004). This pattern typically lead to formation of portal-portal septa surrounding liver lobules, initially preserving connections between central vein and portal tracts, to then progress to biliary cirrhosis, portal hypertension and end-stage liver disease. The critical and peculiar aspect of pathological biliary repair is the appearance of RDCs, a population of cholangiocyte-like epithelial cells of unclear and mixed origin. RDCs orchestrate a very complex process involving several cell types, including macrophage and neutrophils, that first sense biliary injury-derived signals, to involve then MFs, endothelial and HPCs in a joint control of inflammatory and morphogenetic signals (Fabris et al., 2017) that (see section 3.2.3), although prominent in cholangiopathies, are relatively common also in advanced stage of any CLD.

A final mention in the pathogenesis of progressive cholangiopathies should be deserved to the pro-fibrogenic role of intrahepatic accumulation of bile acids, particularly in autoimmune diseases like PBC and PSC. Bile acids contribute by eliciting hepatocyte injury and death (apoptotic and necrotic) (Hofmann and Hagey, 2008; Arndtz and Hirschfield, 2016) as well as through activation of the bile acid/farnesoid X receptor (FXR) (Fickert et al., 2009).

5. Key pathogenic therapeutic targets to affect CLD progression: of cells, mechanisms, signaling pathways and more

Liver fibrosis can be modulated either by negatively affecting CLD progression and/or by positively promoting resolution. The primary target for any therapeutic strategy is represented, whenever possible, by the withdrawal of the etiological agent or condition involved in perpetuating parenchymal damage. The extremely high efficacy of DAA in clearing HBV or HCV infection represents the ideal selective therapy, with abstinence from

alcohol consumption being a plausible option for limiting ALD progression and/or allow regression. For all the other CLD the withdrawal of primary etiology is unfeasible and we lack validated anti-fibrotic drugs for clinical use. In the next sections we will analyze the different strategies and therapeutic targets recently proposed to negatively impact CLD progression.

5.1 Drugs to reduce liver parenchymal injury

This therapeutic strategy is intended to minimize chronic parenchymal injury induced by the primary etiology to potentially prevent inflammation and fibrogenic progression. According to the established involvement of ROS and oxidative stress in progressive CLD, several antioxidants and hepatoprotective agents (some also acting as antioxidants) have been reported to significantly prevent hepatocyte injury and death and/or to limit inflammatory and even fibrosis in rodent models. The list include vitamin E, glutathione, N-acetylcysteine, S-adenosyl-methionine, resveratrol, curcumin, herbal supplements (Sho-Saiko-to, Silymarin, Salvia Miltiorrhiza, etc.), inhibitors of NADPH-oxidase isoforms and many others, as reviewed elsewhere (Weisenkirchen, 2016; Luangmonkong et al., 2018). Unfortunately, administration of these molecules in human patients was either ineffective or associated with very limited or transient efficacy, with few exceptions (reviewed in: Serviddio et al., 2013; Weiskirchen, 2016; Luangmonkong et al., 2018). For example, long-term vitamin E (96 weeks) administration has been reported to be beneficial in a multicenter, randomized, placebo-controlled study performed on non-diabetic NASH patients resulting in histological regression, reduction of liver injury and inflammation, but not fibrosis (Sanyal et al., 2010). Long-term administration (52 weeks) of cysteamine in NAFLD children resulted in significant reductions in serum aminotransferase levels and lobular inflammation but not in an improvement of histological markers (Schwimmer et al., 2016).

A different strategy to reduce parenchymal injury relies on the administration of pan-caspase inhibitors including emricasan and PF-03491390. Emricasan offered interesting results in experimental models of NASH and BDL (Barreyro et al., 2015; Eguchi et al., 2018) and encouraging preliminary results in a short term trial in HCV patients (Pockros et al., 2007). Similar results were described in a longer trial (12 weeks) using PF-03491390 (Shiffman et al., 2010).

5.2 Targeting KC and MoMF as well as mechanisms and signaling pathways underlying their recruitment/activation

According to the major role of KC and MoMFs in CLD progression, pre-clinical studies designed to target these cells have provided interesting results potentially translatable into clinical conditions (Tacke, 2017; Krenkel and Tacke, 2017).

Activation of KC, critical in the early phases of chronic parenchymal injury, can be targeted by affecting gut barrier permeability and gut microbiome (then circulating levels of endotoxin) by modifying bile acid composition or using probiotics, antibiotics or even fecal microbiota transfer (Heymann and Tacke, 2009; Mencin et al., 2009; Ju and Tacke, 2016; Marchesi et al., 2016; Putignani et al., 2016). In murine transgenic models (not translated in humans), this strategy can reduce bacterial translocation and KC activation through TLR4 and improve experimental steatohepatitis (in a CX3CR1-dependent way) (Schneider et al., 2015), fibrosis (TLR4 is expressed by activated HSC/MFs) (Seki et al., 2007) and carcinogenesis (Dapito et al., 2012).

Activation of KC and MoMFs can be also targeted by selonsertib, a selective inhibitor of the serine/threonine kinase ASK1 and downstream signaling pathways, that reduced fibrosis in a phase II multicenter open-label clinical trial on fibrotic NASH patients (Loomba et al., 2016).

Another option is to target chemokine-chemokine receptor interactions that sustain recruitment of monocytes, particularly the prominent CCL2-CCR2 interaction (Marra and Tacke, 2014), as shown in murine models using monoclonal antibodies against chemokines or related receptor(s), receptor antagonists or by inhibiting chemokines with aptamer molecules or small molecule inhibitors (Baeck et al., 2012; Baeck et al., 2014; Oberthur et al., 2015). Accordingly, the dual oral CCR2/CCR5 antagonist cenicriviroc, through inhibition of MoMF recruitment, is antifibrotic in murine NASH models (Krenkel et al., 2018) and in human fibrotic NASH patients in a phase IIb trial (Friedman et al., 2018a). Another emerging anti-fibrotic option is to target KC and/or MoMF through systemic administration of liposomes, hard-shell microbubbles and polymers working as delivery carriers (Bartneck et al., 2014; Ergen et al., 2017) for drugs, as shown for dexamethasone (Melgert et al., 2001; Bartneck et al., 2015), and potentially siRNA for gene silencing, specific inhibitors of inflammatory signaling or enhancers of autophagy.

Alternatively, KC and MoMF have been targeted using carbohydrate molecules like GR-MD-02 (galactoarabino-rhamnogalacturonan) or GM-CT-01 (galactomannan) that are able to inhibit galectin-3 which mediate inflammatory macrophage functions in CLD. These molecules significantly inhibited experimental hepatic fibrosis (Traber et al., 2013) and GR-MD-02 has been recently successfully tested for safety, pharmacokinetics and exploratory pharmacodynamics markers in a phase 1 clinical trial in fibrotic NASH patients (Harrison et al., 2016).

Finally, some years ago a study reported that the adoptive transfer of syngeneic ex-vivo polarized restorative macrophages decreased fibrosis and improved regeneration and liver function in a murine model of CLD (Thomas et al., 2011). Although it is technically possible to obtain CD14⁺ monocytes from CLD patients to be differentiated into resolution macrophages (Moore et al., 2015), repeated transplantation of bone marrow-derived stem cells in cirrhotic patients did not afford clinical benefits (King et al., 2015) and the potential

use of macrophage-based therapies for CLD patients remains undetermined (Forbes et al., 2015).

5.3 Targeting MFs and/or mechanisms and signaling pathways underlying their activation and pro-fibrogenic role

Our present knowledge on the mechanisms underlying activation of pro-fibrogenic MFs mostly comes from studies on HSC, as recently extensively and authoritatively reviewed (Lee et al, 2015; Higashi et al., 2017; Tsuchida and Friedman, 2017) and summarized in Figure 1. The next sections briefly recapitulate most relevant and emerging concepts, with a focus on those mechanisms which have been translated into clinical conditions.

5.3.1 Targeting extracellular events leading to HSC activation

We have already described some of the potentially targetable extracellular events relevant to activate HSC, including hepatocyte injury and death, the involvement of innate and adaptive immune cells, chronic infection by HBV and HCV, metabolic dysregulation and gut dysbiosis. We have also mentioned the putative role of HPC and RDC, particularly prominent in progressive NAFLD and biliary like fibrosis. It should be noted that both HPC and RDC are potentially targetable since they uniquely express the integrin $\alpha\beta6$, which in addition to bind fibronectin and tenascin-C, can act as co-activator of latent TGF β 1. The block of this integrin using either a small molecule inhibitor or a specific antibody, resulted in attenuation of biliary and non-biliary experimental fibrosis (Patsenker et al., 2008; Popov et al., 2008; Peng et al., 2016). In addition, activation of HPC has been reported to result in a severe and TNF-like weak activator (TWEAK)-dependent pro-fibrogenic response that can be counteracted by using a specific TWEAK-neutralizing antibody (Kuramitsu et al., 2013). However, these approaches have not been translated into clinical trials. Similarly, no human data is available concerning the specific targeting of LSEC, which are known to maintain HSC in a quiescent phenotype (Poisson et al., 2017) although positive results

(reversion of HSC activation and reduction of fibrosis) were obtained in an experimental study using the soluble guanylate cyclase activator BAY-60-2770 (Xie et al., 2012).

HSC activation can also relies on the contact with altered ECM (shifting from basal-like towards fibrillary ECM) through integrin-mediated signals (Henderson et al., 2013) or to favor HSC activation dependent on several peptide mediators (PDGF, FGF, HGF, VEGF) that remain entrapped in the altered ECM (Lee et al., 2015). Along these lines, reduction of experimental fibrosis has been obtained using a monoclonal antibody against Lysyl-oxidase like-2 (LOXL2), an enzyme expressed by HSC that catalyzes crosslinking of collagens and elastins (Barry-Hamilton et al., 2010). This approach has been recently translated in humans using the humanized anti-LOXL2 antibody Simtuzumab (G6-6624) that was first analyzed for safety in fibrotic HCV patients first in an open label trial (Meissner et al., 2016). Simtuzumab was then tested in phase II trials for either fibrotic (NCT01672866) or cirrhotic (NCT01672879) NASH patients, but this therapeutic programme has been recently halted due to simtuzumab non efficacy.

5.3.2 Targeting mechanisms resulting in dysregulation of critical molecular pathways in activated HSC or MFs

5.3.2.1 Pathways elicited by ligand-receptor interactions

Several peptide growth factors, by interacting with their cognate receptor(s), can affect and sustain one or more of the phenotypic responses of activated HSC and/or MFs (Figure 1). Pre-clinical studies, using genetically manipulated mice, pharmacological inhibitors, neutralizing antibodies, adenoviral vectors or siRNAs, have confirmed that to affect these ligand-receptor interactions is effective in vitro as well as in reducing experimental liver fibrosis (Higashi et al., 2017; Tsuchida et al., 2017). Disappointingly, only a limited number of theoretically available approaches has been translated into clinical trials and few studies have reported some benefit in CLD patients.

A typical example is represented by the signaling pathways elicited by either TGF β 1 or PDGF, growth factors acting on MFs but also released by these cells. Despite knowledge on molecular mechanisms involved and positive results obtained by targeting these pathways in animal experiments (Lee et al., 2015; Higashi et al., 2017; Tsuchida et al., 2017; Cannito et al., 2017b) no drug or procedure has been specifically validated for human progressive CLD, differently from what reported for fresolimumab for glomerular sclerosis or pirfenidone and nintedanib for idiopathic pulmonary fibrosis (IPF) (Cannito et al., 2017b). Similar considerations can be applied to other ligand-receptor – induced signaling pathways, including those related to HGF, EGF/EGFR, VEGF/VEGFR, Wnt/ β -catenin, Hedgehog, endotelins, cannabinoids, adipokines, retinoid and vitamin D receptors, integrins and TLRs (Higashi et al., 2017; Tsuchida and Friedman, 2017).

As a pertinent example, connective tissue growth factor (CTGF) is believed to be critical in mediating TGF β 1 pro-fibrogenic effects (Jun and Lau, 2011) and experimental targeting of CTGF can affect HSC activation and inhibit experimental fibrosis (Uchio et al., 2004; Li et al., 2006; Hao et al., 2014). An antibody against CTGF has been tested in a clinical trial on HBV patients in addition to entecavir (NCT01217632) that was perhaps terminated because of the potent effect of the arm with entecavir alone (Hauff et al., 2015).

Another example concern the targeting of renin-angiotensin system employing losartan, an inhibitor of the receptor for angiotensin II AT1R, which is highly expressed by activated HSC, with angiotensin II stimulating the proliferation, migration, contractility and TGF β 1 and collagen I expression in these cells (Bataller et al., 2005; Moreno and Bataller, 2008). Losartan has been reported to reduce fibrosis in experimental animals (Yang et al., 2005; Moreno et al., 2010) and, possibly, in HCV patients (Salama et al., 2016) through modulation of non-phagocytic NADPH-oxidase and profibrogenic genes (Colmenero et al., 2009).

5.3.2.2 Nuclear receptor signaling pathways

Several nuclear transcription factor receptors, including peroxisome proliferator-activated receptor (PPAR)- γ and PPAR- δ , farnesoid X receptor (FXR), liver X receptor (LXR), vitamin D receptor (VDR), nuclear receptor subfamily 4 group A member 1 (NR4A1) and nuclear receptor subfamily 1 group D member 1 (REV-ERB α) are expressed by HSC (Tsuchida and Friedman, 2017). These nuclear receptors, which regulate energetic fluxes and metabolic pathways, are dysregulated in CLD, particularly in progressive NAFLD (Machado et al., 2014; Wang et al., 2015), and have been reported to inhibit HSC activation and fibrosis progression. Interestingly, drugs affecting nuclear receptors, particularly PPARs and FXR, have been translated into clinical trials.

The members of the PPARs family (PPAR- α , PPAR- γ , PPAR- δ) are activated by fatty acids and operate by forming an heterodimer with retinoid X receptor (RXR) to act on PPAR responsive elements. PPAR- α is believed to afford protection in progressive NAFLD but although its expression was inversely correlated with critical parameters (insulin resistance, severity of steatosis, presence of steatohepatitis and fibrosis), PPAR- α agonists were ineffective in clinical trials (Tailleux et al., 2012; Ballestri et al., 2016). More interesting are the data concerning PPAR- γ activation that reverts activated HSCs to a quiescent phenotype and down-regulate in these cells the expression of α SMA, type I collagen and TGF β (Hazra et al., 2004), with murine models confirming the relevance of PPAR- γ and PPAR- δ for fibrosis development (Moran-Salvador et al., 2013; Iwaisako et al., 2012). These results led to test in clinical trials for NASH patients thiazolidinediones like pioglitazone and rosiglitazone that in a phase III trial improved steatosis and lobular inflammation but not fibrosis (Sanyal et al., 2010). Along these lines, GFT505 (elafibranor), a dual PPAR α -PPAR δ agonist, reduced steatosis, inflammation and fibrosis in animal models (Staels et al., 2013) and was employed in large randomized clinical trial showing resolution of NASH without worsening of fibrosis (Ratziu et al., 2016), with a phase III trial using elafibranor actually ongoing (NCT02704403).

FXR is another example of nuclear receptors inhibiting HSC activation, with FXR deficiency in mice resulting in a worsening of hepatic inflammation and fibrosis (Kong et al., 2009). Orally available FXR agonists are currently being tested in clinical trials and a first relevant example is obeticholic acid which has been employed in a large randomized phase II clinical trial on NASH patients resulting in a remarkable improvement of NAFLD activity score and fibrosis stage (Neuschwander-Tetri et al., 2015). At present two obeticholic acid - based phase III trials are on-going to verify the ability of the drug to improve liver fibrosis without worsening NASH (NCT02548351, NCT03439254). Obeticholic acid has been also positively employed in clinical trials performed on PBC patients (Hirschfield et al., 2015; Nevens et al., 2016). Other FXR agonists have been employed or are currently being tested in clinical trials, as recently reviewed (Wiest et al., 2017; Friedman et al., 2018b): i) GS-9674, tested for safety, tolerability and pharmacokinetics in phase I trials (NCT02854605, NCT02808312) and then used in phase II trials on NAFLD/NASH patients (NCT02781584) on in non-cirrhotic PSC patients (NCT02943460); ii) other FXR agonists being tested in phase II trials on NASH patients include LJM452 or tropifexor (NCT02855164), EDP-505 (NCT03421431) and LMB-763 (NCT02913105).

5.3.3 Targeting transcription factors and epigenetic transcriptional dysregulation

Transcription factors (TFs) and epigenetic transcriptional dysregulation have a critical role in modulating HSC activation, as shown by pre-clinical studies. This include TFs that contribute to up-regulate activation of HSC like myocardin-related transcription factor A (MRTF-A), sex-determining region Y-box 9 (SOX9), aryl hydrocarbon receptor (AhR), Yes associated protein (YAP) and G α -interacting vesicle-associated protein (GIV). Inhibition or genetic silencing of these factors (that up-regulate directly or indirectly critical profibrogenic genes) results in a significant reduction of fibrosis in experimental models. Other TFs

negatively modulate/repress pro-fibrogenic genes and HSC activation, including Kruppel-like factors (KLF6 and -2), GATA binding protein 4 (GATA4), NR4A1 and NR4A2; the up-regulation or stimulation of the activity of these TFs should prevent fibrosis whereas their downregulation exacerbates fibrosis (see Higashi et al., 2017; Tsuchida and Friedman, 2017). For example, KLF6 represses collagen I and PDGFR β expression and leads to HSC apoptosis, with KLF6 down-regulation exacerbating experimental liver fibrosis (Ghiassi-Nejad et al., 2013). Concerning KLF2, an experimental study has revealed that statins up-regulate KLF2 expression in LSEC leading to HSC quiescence through a NO and guanylate cyclase paracrine signaling mechanism (Marrone et al., 2013).

Concerning epigenetic transcriptional dysregulation in HSC activation, studies on quiescent and activated human HSC have identified 212 profibrogenic miRNAs overexpressed in activated HSC (Coll et al., 2015) including miR- 21, miR- 27, mirR-125, miR-195, miR-199a, miR-199b, miR- 221 and miR-222, each of these miRNA being able to stimulate proliferation, collagen synthesis and/or migration. By contrast, 47 antifibrotic miRNAs are down-regulated in activated HSC, including miR15b, miR-16, miR-29, miR-122, miR-133b and miR-200a (Coll et al., 2015).

DNA methylation and histone modification have also been reported to contribute to HSC activation (Tsuchida and Friedman, 2017; Higashi et al. 2017). For example, MRTF-A can recruit a histone methyltransferase complex that lead to key histone modifications allowing transcriptional activation of the promoters of fibrogenic genes (Tian W et al., 2016). In addition, methylation of cytosine–phosphoguanine (CpG) dinucleotides by Methyl-CpG binding protein 2 (MECP2) and histone-lysine *N*-methyltransferase enhancer of zeste homolog 2 (EZH2) have been reported to repress PPAR- γ transcription to promote HSC activation and then sustain fibrosis (Mann et al., 2010).

5.3.4 Targeting pathways related to cellular stress conditions or altered metabolism

Autophagy is believed to contribute to HSC activation by generating FA from cleavage of retinyl esters within cytoplasmic droplets. The relationships between autophagy and fibrogenesis has been validated in experiments employing mice carrying HSC-specific deficiency of autophagy-related protein 7 (ATG7) (Hernandez-Gea et al., 2012) or using the autophagy inhibitor bafilomycin A1 in cultured HSC (Thoen et al., 2011).

ER stress signals can induce fibrogenic activation of HSC and this is linked to increased autophagy, as shown by pre-clinical studies employing targeted lentiviral delivery of 78 kDa glucose-regulated protein (GRP78), blockade of the inositol-requiring enzyme 1 α (IRE1 α) pathway or modulating PKR-like endoplasmic reticulum kinase (PERK) (Hernandez-Gea et al., 2013; Koo et al., 2016). Similarly, ER stress and apoptosis in HSC are exacerbated following inhibition of autophagy in HSC depleted for heat shock protein 47 (HSP47), a molecular chaperone essential for collagen type I maturation and secretion (Kawasaki et al., 2015). The latter strategy is at present investigated in a clinical trial that is testing for efficacy Vitamin-A- coupled lipid nanoparticles containing siRNA against HSP47 (NCT02227459). Still related to ER stress, it has been reported that JNK signaling, particularly JNK1-dependent in HSC contributes to fibrogenesis, as shown in experimental models of fibrosis by employing a JNK-inhibitor (Kluwe et al., 2010).

As mentioned, quiescent HSC contain vitamin A and retinoids in their cytoplasmic droplets and loss of these lipid droplets is an established, feature of HSC activation. Accordingly, isoform 3 of alcohol dehydrogenase (ADH3), involved in retinol metabolism, has important roles in promoting liver fibrosis by enhancing HSC activation and inhibiting NK cytotoxicity against HSC, as shown by specific ADH3 inhibition or genetic depletion (Yi et al., 2014).

High cholesterol levels can additionally support HSC activation as shown in dietary murine models of fibrosis (Tomita et al., 2014) and by studies indicating that accumulation of free in HSC sensitizes the cells to TGF β -induced activation through enhancement of TLR4-

mediated downregulation of the TGF β pseudoreceptor BAMBI (Teratani et al., 2012). Accordingly, statins or ezetimibe have been reported to reverse hepatic accumulation of free cholesterol and to attenuate steatohepatitis and fibrosis in a murine model of NASH (Van Rooyen et al., 2013), but this potentially translatable approach has not yet been assessed in large cohorts of patients.

5.4 To promote resolution of fibrosis.

Three main strategies may promote resolution of liver fibrosis: i) to induce specific elimination of pro-fibrogenic cells or, alternatively, their reversion or senescence, ii) to increase ECM degradation or iii) to transplant bone marrow-derived cells (i.e., macrophages).

HSC are resistant to cell death through up-regulation of survival and NF- κ B-dependent signals (Novo et al., 2006; Lee et al., 2015) but HSC specific killing has been reported in cultured HSC and/or in vivo models by administering gliotoxin, the NF- κ B inhibitor BAY 11-7082 or the proteasome inhibitors MG-132 and bortezomib (reviewed in Weiskirchen, 2016; Higashi et al., 2017). Apoptotic and autophagic cell death of HSC has been induced either by nilotinib, through inhibition of histone deacetylases (Shaker et al., 2013), or by sorafenib through JNK/Akt pathway (Hao et al., 2016).

HSC deactivation/reversion can be obtained following withdrawal of etiological agents, although reverted cells may be re-activated if exposed to fibrogenic stimuli (Kisseleva et al., 2012; Troeger et al., 2012) and experimental transcriptional reprogramming of HSC into hepatocyte-like cells can result in fibrosis reduction (Song et al., 2016).

Senescence of activated HSC has been achieved experimentally using a number of different compounds, including curcumin, cysteine-rich protein 61 (CCN-Cyr61) or OSU03012, a celecoxib derivative (reviewed in Higashi et al., 2017).

Alternatively, experimental studies were designed to promote degradation of the collagen-rich ECM by using TIMPs antagonists (Parsons et al., 2004) or by monoclonal antibody against LOXL2 that inhibit its collagen cross-linking activity (Barry-Hamilton et al., 2010). Two clinical trials explored the latter strategy using the humanized antibody Simtuzumab for either fibrotic (NCT01672866) or cirrhotic (NCT01672879) NASH patients. However, as previously mentioned, the Simtuzumab strategy was abandoned due to its lack of efficacy. Finally, a strategy based on the transplantation of bone marrow progenitor cells, particularly “resolutive macrophages”, is under evaluation. This approach is aimed at promoting fibrillary ECM degradation and ultimately promote regeneration (Thomas et al., 2011; Moore et al., 2015).

6. The clinical evaluation of fibrosis progression in chronic liver diseases

6.1 Liver biopsy for the evaluation of “disease severity”

Liver fibrosis is the key determinant of the evolution of CLD to cirrhosis and of the development of clinical complications typical of the advanced stage of the disease. In spite of the mechanistic complexity inherent in the progression of the hepatic fibrosis, its clinical evaluation has been based on the establishment of identifiable endpoints and particularly on the histopathological assessment of liver tissue fibrosis. Accordingly, the current interpretation of CLD progression is based on the evaluation of what is defined ‘disease severity’, which is a potentially misleading concept when indicating just the extent of tissue fibrosis (Germani et al., 2011). In general, all CLDs are characterized by a long clinical course practically devoid of symptoms, and become clearly severe diseases when they reach the stage of decompensated cirrhosis, that is, a condition characterized by life-threatening events due to portal hypertension (PH) and overt hepatocellular failure. However, the categorization in fibrosis stages employing semi-quantitative staging

systems is a clinical compromise that does not reflect the biological complexity of disease progression. Ideally, the progression of CLD should be analyzed according to etiology-driven mechanisms in order to identify more accurate diagnostic endpoints and predictive indexes guiding a more effective clinical management (Quaglia et al., 2016).

The introduction in the 1980's of semi-quantitative scoring systems and the relative definition of grading (necro-inflammatory activity) and staging (fibrosis) for the evaluation of liver biopsy was aimed at standardizing the interpretation and facilitate communication between pathologists and clinicians. However, this change in methodology did not overcome the impact of interpretative problems, such as reproducibility, intra-observer and inter-observer agreement. For these and other reasons (sampling error, lack of adequate standards, etc.), the value of liver biopsy as a gold standard has become questionable for clinicians and pathologists particularly when biopsy started to be used almost exclusively for assessing the extent of tissue fibrosis (Rosselli et. al, 2013).

Taken together, these considerations promote a thoughtful re-evaluation of the role of liver biopsy in modern Hepatology. With a vision at implementing the evaluation of disease progression in CLD, it has been proposed to replace the semi-quantitative scoring systems with a standardized morphometric analysis of liver tissue fibrosis. Indeed, computer-assisted morphometry could provide a quantitative measure of hepatic fibrosis on a continuous scale and, when adequately standardized, greatly reduces intra-observer and inter-observer variability. Morphometric analysis using the Collagen Proportionate Area (CPA) system has demonstrated an excellent positive correlation between the amount of fibrosis in a cirrhotic liver and the relative hepatic vein pressure gradient (HVPG) and liver tissue stiffness.

While we are reconsidering the role of liver biopsy, it is increasingly clear that the assessment of disease progression and regression should be able to conjugate morpho-imaging with the detection of key pathophysiological events (fibrogenesis, angiogenesis,

and liver regeneration) and the assessment of different aspects of liver function. Key progression elements, such as hepatocellular necrosis, apoptosis and regeneration, ductular reaction, increasing tissue hypoxia and endothelial dysfunction, scar contraction due to an unbalanced presence of vasoconstrictors, pre-neoplastic features are not yet fully considered although they could represent optimal tissue biomarkers for the development of advanced bio-imaging technologies. Additionally, advanced comprehensive liver imaging could resolve the problem of sampling error caused by the often non uniform distribution of the fibrogenic process especially in early phases of CLD (Hytiroglou et al., 2012).

6.2 The non-invasive evaluation of liver fibrosis.

The introduction of different non-invasive measures for assessing the fibrogenic evolution of CLD has provided major changes in clinical practice over the past 15 years and has not only resulted in earlier detection of patients with hepatic fibrosis, but also new models for the stratification, prognostication and treatment of patients with chronic liver diseases (EASL-ALEH Clinical Practice Guidelines, 2015; de Franchis, 2015). The tools available for non-invasive assessment of fibrosis range from simple scores calculated from routine laboratory parameters or more complex serum biomarkers based on the determination of circulating components relative to the accumulation and remodelling of the extracellular matrix occurring during the fibrogenic process, to elastography techniques to measure liver stiffness. The aim of all these methodologies is to overcome the disadvantages inherent in liver biopsy/histology and hepatic venous pressure gradient (HVPG) measurement and, ultimately, to reduce the need of these invasive approaches. Importantly, the use of non-invasive tests has in several instances progressed beyond the initial purpose of assessing the extent of fibrosis, to predicting the consequences of chronic liver disease including portal hypertensive complications and the development of HCC (Rosselli et. al, 2013).

In everyday clinical practice, non-invasive tests are employed to detect two histopathological endpoints: significant fibrosis and cirrhosis. The definition of these endpoints is dependent on the histological scoring system used. The METAVIR or Ishak systems have been used in chronic viral hepatitis and have formed the basis for validation of non-invasive tests. Significant fibrosis refers to a METAVIR score of F2 or greater (Ishak ≥ 3), whereas METAVIR F4 (Ishak ≥ 5) denotes cirrhosis. The presence of significant fibrosis represents a clear indicator for the tendency of disease progression to cirrhosis and end-stage liver disease. Accordingly, any available medical treatment and/or change in lifestyle become mandatorily indicated. The diagnosis of cirrhosis defines a poorer prognostic group at risk of developing complications of chronic liver disease. These patients not only need treatment of the underlying cause but also surveillance for complications of portal hypertension and HCC. The development of either of these histopathological endpoints often precedes any overt clinical features, thus highlighting the important clinical role of invasive or non-invasive tests for liver fibrosis (Trautwein et al., 2015).

In the past decade, particularly in reason of the increasing attention to the extraordinary high incidence of NAFLD and the relative potentially evolutive for NASH, several strategies for risk stratification of fibrosis progression in patient with NAFLD have been proposed. Figure 2 illustrates a simplified scheme employing non-invasive method whose application is initiated in primary care with the utilization of simple algorithms derived from standard blood tests and clinical features. Along these lines, it is increasingly evident that an effective risk assessment and the establishment of effective referral pathways depend upon the awareness and the competence of primary care physicians.

7. Liver fibrosis and antifibrotic strategies: clinical endpoints

The possibility of preventing the progression of liver fibrosis in CLD and/or to induce fibrosis regression is implicit in the concept of liver fibrosis which is up to a certain extent a highly dynamic process (Ramachandran and Henderson, 2016). Reversibility of fibrosis has been demonstrated in nearly all chronic liver diseases, following removal of the causative agent. In particular, large clinical trials in patients with chronic viral hepatitis with histological evaluation of fibrosis before and after antiviral therapy, have provided convincing data that fibrosis can regress even in patients with advanced disease. This is clinically very relevant since improvements in liver fibrosis are associated with better clinical outcomes and with a significant increase in 10-year survival. Therefore, effective antifibrotic treatments would be highly beneficial, even in patients with advanced fibrotic liver disease (Trautwein et al., 2015).

Overall, the success of antiviral treatments in blocking the fibrogenic progression of chronic liver disease has provided key information on the natural history of fibrosis regression, and has established important benchmarks and targets for antifibrotic drugs.

When discussing end-points for antifibrotic treatment is important to make two major distinctions. The first is relative to the etiology of CLD and is based on the evidence that the primary cause of chronic liver damage influences the prevalent pro-fibrogenic mechanisms and the pattern of fibrotic evolution of the disease (Pinzani and Rombouts, 2004; Böttcher and Pinzani, 2017). This is likely to have important implications especially in the early phases of the fibrogenic process when the possibility of fibrosis regression is higher also because of the absence or scarce relevance of associated changes in the tissue architecture such as significant neo-angiogenesis (Bocca et al., 2015).

The second distinction is relative to the stage of fibrotic evolution, which should not be necessarily identified with the clinical stage of the disease. Indeed, the gross classification in pre-cirrhotic and cirrhotic phase of the disease has important implications in terms of

end points. Definitively, while there is consistent evidence of the reversibility of fibrosis in non cirrhotic liver, the determinants of fibrosis regression in cirrhosis are not sufficiently clear, and the so-called “point of no return” in cirrhosis is not clearly established, both in morphologic and functional terms. Tissue fibrosis within a cirrhotic liver is characterized by an increasing cross-linking of high-density fibrillar collagens (e.g., collagens I and III) as well as an increased expression and cross-linking of elastin (Schuppan et al., 2018b). Collagen and elastin crosslinking enhances the resistance of established scar tissue to degradation. In addition, the ongoing experience on the treatment of HCV in patient with cirrhosis has also highlighted the fact that removing the cause of chronic liver damage and obtaining a SVR does not immediately result in the abrogation of the fibrogenic process that may further progress as suggested by the aggravation of portal hypertension and the persistent risk of developing hepatocellular carcinoma (Di Marco et al., 2016; Afdhal et al., 2017). Thus, removing the causative pathogen may be not sufficient to reliably improve clinical outcomes in all patients with liver fibrosis, again highlighting the urgent need for directly acting, potent antifibrotic therapies to be employed before, during and after the establishment of antiviral therapy.

In spite of the large amount of information on the causes and the mechanisms responsible for the fibrogenic evolution of CLD and the large success of preclinical tests in animal models there is not yet a pharmacological compound or a biotechnological strategy that can be employed in clinical practice. Several clinical trials investigating the direct or indirect antifibrotic effect of different compounds are currently ongoing in Europe (<https://www.clinicaltrialsregister.eu/ctr-search/search?query=Liver+Fibrosis>) and USA (<https://clinicaltrials.gov/ct2/results?recrs=&cond=liver+fibrosis&term=&cntry=US&state=&city=&dist=>). It is very relevant that the lists in these websites include also clinical trial on the diagnostic accuracy of different types of measures for a more accurate patient stratification and the monitoring of the antifibrotic effects of different agents. Regardless,

it remains to be clarified which fibrosis-related endpoints are effective predictors of clinical outcomes and which surrogate markers could be employed. If the trial is conducted in patients with advanced fibrosis/cirrhosis, the hepatic venous pressure gradient (HVPG), with HVPG > 10 mm indicating an increased risk of clinical decompensation, could help identifying patients responding or not to the treatment. However, the clinical value of variations in HVPG, which for values < 10 mmHg are known to have a linear relationship with tissue fibrosis, following treatment clearly lacks of precision and can only provide a generic insight. Also, in conditions of HVPG <10 mmHg, some additional support derive by the use of liver stiffness as a complement or a surrogate of HVPG measurement (Vizzutti et al, 2007).

Conclusions

The research field of liver fibrosis has reached a stage of maturity and the expectations for a more effective clinical management have become more and more realistic. Intensive basic research in liver fibrosis, started more than thirty years ago, has somehow progressively prompted the attention of clinicians towards this common denominator of any liver disease characterized by chronic tissue damage. Accordingly, the past 20 years have been characterized by parallel advancements in the identification of targets for anti-fibrotic therapy and in non-invasive methodologies and biomarkers for assessing the fibrotic progression of CLD and potentially the response to treatment. Currently, the discussion is centered on which stage of fibrotic evolution would have the maximal benefit from antifibrotic therapy and which are the relative realistic clinical endpoints. The large number of clinical trials investigating the direct or indirect effect of many antifibrotic drugs will certainly lead to the possibility of using some of these agents in the near future and this will greatly affect the landscape of Hepatology as well as other medical specialties.

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Figure legends

Figure 1. Major signaling pathways , molecules and mechanisms regulating HSC activation. HSC activation is regulated by a number of pathways and signaling molecules or events that can either sustain or inhibit activation of HSC and then their proliferative and/or profibrogenic responses. Regulation of HSC activation can operate through interaction of peptide ligands with their cognate receptors or through the action of miRNAs, nuclear receptors, transcription factors and epigenetic changes. Other events and mechanisms can sustain HSC activation , including autophagy, which in turn is linked to ER stress, oxidative stress and loss of retinoids. Green and red font indicate signals and pathways positively or negatively affecting HSC activation, respectively.

Figure 2. Diagnostic flow-chart for the non-invasive assessment of liver fibrosis.

Figure 1

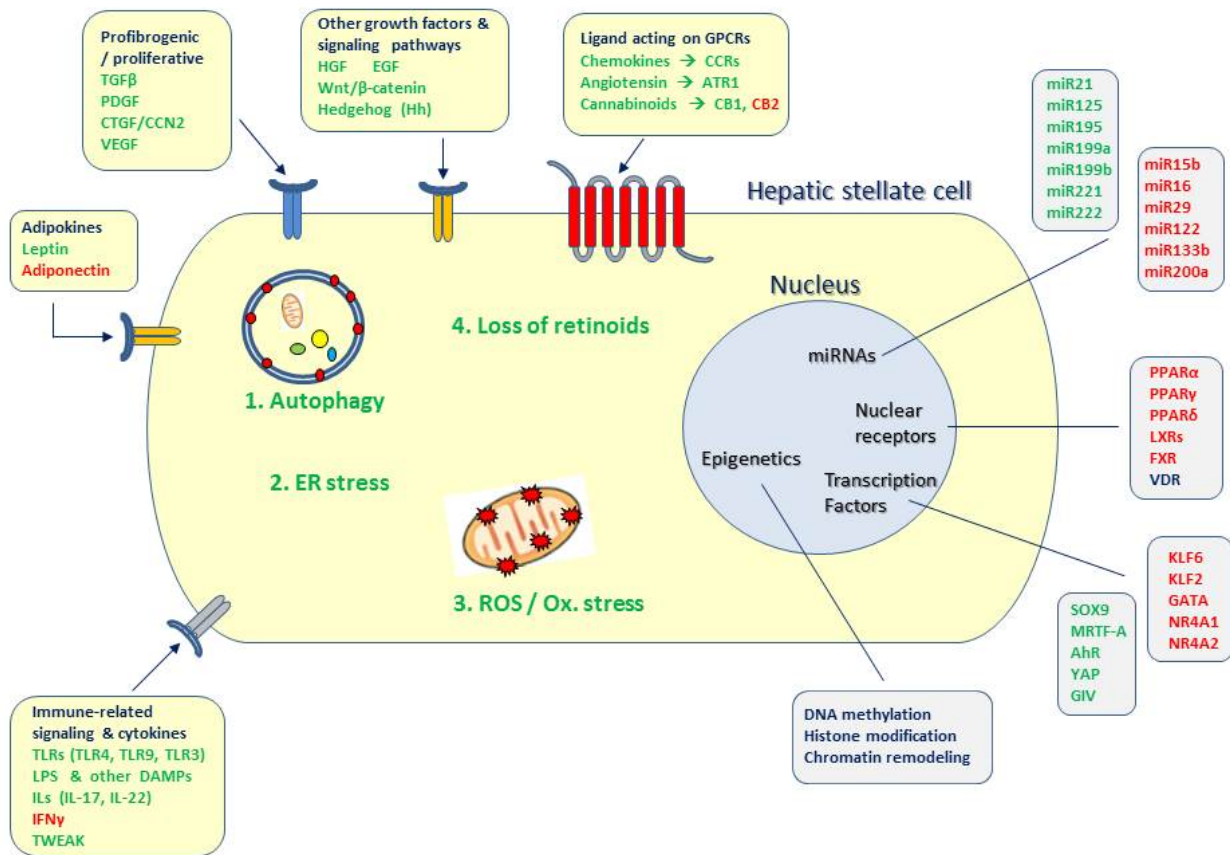


Figure 2

