**Liver fibrogenesis: un update on established and emerging basic concepts**

# LIVER FIBROGENESIS: UN UPDATE ON ESTABLISHED AND EMERGING BASIC CONCEPTS

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**Author contribution**: All Authors were involved in conceptualization, writing-original draft preparation as well as reviewing and editing.

**Conflict of interest statement.** Authors involved in the present study declare that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

**Highlights**

- Liver fibrogenesis is a major driving force for chronic liver disease progression (CLD)
- Myofibroblasts, macrophages and other cell populations are involved in fibrogenesis
- Unravelling molecular, cellular and tissue mechanisms is critical to design novel therapies
- Etiology-independent and etiology dependent mechanisms sustain fibrogenesis
- Genetic and epigenetic factors are emerging as critical for CLD progression
- Hypoxia, hypoxia-inducible factors and related mediators play a role in CLD progression

*Abbreviations used:*

ACC1, Acetyl-CoA carboxylase 1; ALD, alcoholic liver disease; AMPK, AMP-activated protein kinase; Ang-II, angiopoietin II; ANGPTL3, angiopoietin-related protein 3; ANGLPTL4, angiopoietin like 4; α-SMA, α-smooth-muscle actin; ASH, alcoholic steatohepatitis; ATF6, activating transcription factor 6; ATG, autophagy related protein; BAs, bile acids; bFGF, basic fibroblast growth factor; BAX, BCL-2-associated X protein; Bcl2, B-cell lymphoma/leukemia-2; bHLH-PAS, basic helix–loop–helix Per–Arnt–Sim; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; BiP, immunoglobulin heavy chain-binding protein; CCL, C-C- chemokine ligand; CCR, C-C-chemokine receptor; CHC, chronic hepatitis C; CHOP, CCAAT-enhancer-binding protein homologous protein; CXCL, C-X-C chemokine ligand; CXCR, C-X-C-chemokine receptor; CLD, chronic liver diseases; CPA, collagen proportionate area; CREB, cAMP response element binding protein; CTGF, connective tissue growth factor; CYP2E1, cytochrome P450 2E1; DAMPs, damage-associated molecular patterns; DC, ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; ER, endoplasmic reticulum; ERK, extracellular signal - regulated kinase; ET-1, endothelin-1; EVs, extracellular vesicles; FABP4, fatty acid binding protein 4; FFA, free fatty acid; Fiaf, fasting-induced adipocyte factor; FXR, farnesoid X receptor; GCKR, glucokinase receptor protein; GRP78, 78 kDa glucose-regulated protein; GRP94, 94 kDa glucose-regulated protein; GWAS, genome-wide associated studies; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; HIF, hypoxia inducible factor; HNRNPA1, heterogeneous nuclear ribonucleoprotein A1; HPCs, hepatic progenitor cells; HRGP, histidine-rich glycoprotein; HRE, hypoxia responsive elements; HSC, hepatic stellate cells; HSC-MFs, activated, myofibroblast-like, hepatic stellate cells; HSP, heat shock protein; ICAM1, intercellular adhesion molecule 1; IL, interleukin; IFNγ, interferon-γ; iTCR, invariant T cell receptor; IRE1α, inositol requiring protein 1α; JNK1/2, isoforms 1 and 2 of c-Jun-NH2-kinases; KC, kupffer cells; LDLR, low-density lipoprotein receptor; LIFRβ, leukemia inhibitor factor receptor β; LOX2, lisyl oxidase 2; Ly6C, lymphocyte antigen 6 complex, locus C1; LPS, lipopolysaccharide; MAIT, mucosal-activated invariant T; MAPK, mitogen-activated protein kinase; MBOAT7, membrane bound O-acyltransferase domain

containing 7-transmembrane channel-like 4; MDM, monocyte-derived macrophage; MERTK, MER protocol-oncogene, tyrosine kinase; MFs, myofibroblasts; miRNAs, microRNAs; MMP, metalloprotease; MnSOD, Manganese-dependent superoxide dismutase; mTOR, mammalian target of rapamycin; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-kB, nuclear factor kB; NK, natural killer; NKT, natural killer T; NLR, NOD-like receptor; NLRP3, NOD-like receptor family, pyrin domain containing 3; NOX, NADPH oxidase; OSM, Oncostatin M; OSMRβ, Oncostatin M receptor β; PAMPs, pathogen-associated molecular patterns; PBC, primary biliary cholangitis; PDGF, platelet-derived growth factor; PERK, PKR-like endoplasmic reticulum kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLOD2, procollagen-lysine,2-oxoglutarate 5-dioxygenase 2; PNPLA3, patatin-like phospholipase domain containing-3; PPAR-γ, peroxisome proliferator-activated receptor-γ; PHD, prolyl hydroxylase; PSC, primary sclerosing cholangitis; PUMA, p53 upregulated modulator of apoptosis; RDC, reactive ductular cells; RedoxRI, redox-related reactive intermediates; REDD1, regulated in development and DNA damage responses 1; ROS, reactive oxygen species; SCFAs, short chain fatty acids; SECs, sinusoidal endothelial cells; SOCS, suppressor of cytokine signalling; STAT, signal transducer and activator of transcription; TG, triglycerides; TGFβ1, transforming growth factor β1; TGFß2, transforming growth factor β2; Tie2, angiopoietin I receptor; TIMP, tissue inhibitor of metalloproteases; TLR, toll-like receptor; TNF, tumor necrosis factor α; TM6SF2, transmembrane 6 superfamily member 2; TSG101, tumor susceptibility gene 101; TWIST1, twist-related protein 1; UCP2, uncoupling protein 2; UPR, unfolded protein response; VCAM1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor type 2; VLDL, very low density lipoprotein; VHL, von Hippel-Lindau protein; XBP1, X-box binding protein 1.

**Abstract**

Liver fibrogenesis is defined as a dynamic and highly integrated process occurring during chronic injury to liver parenchyma that can result in excess deposition of extracellular matrix (ECM) components (i.e., liver fibrosis). Liver fibrogenesis, together with chronic inflammatory response, is then primarily involved in the progression of chronic liver diseases (CLD) irrespective of the specific etiology. In the present review we will first offer a synthetic and updated overview of major basic concepts in relation to the role of myofibroblasts (MFs), macrophages and other hepatic cell populations involved in CLD to then offer an overview of established and emerging issues and mechanisms that have been proposed to favor and/or promote CLD progression. A special focus will be dedicated to selected issues that include emerging features in the field of cholangiopathies, the emerging role of genetic and epigenetic factors as well as of hypoxia, hypoxia-inducible factors (HIFs) and related mediators.

**Keywords** (max 6)

Liver fibrogenesis, myofibroblasts, macrophages, cholangiopaties, genetic factors, hypoxia-inducible factors.

## 1. Introduction

### 1.1 Liver fibrogenesis, liver fibrosis and chronic liver disease progression

Liver fibrogenesis is a dynamic and highly integrated fundamental biological process that, following chronic injury to hepatic parenchyma and in an attempt to limit related consequences, leads to excess deposition of extracellular matrix (ECM) components (i.e., liver fibrosis) [1-3]. Liver fibrogenesis is primarily involved in the progression of chronic liver diseases (CLD) as mainly induced by: i) chronic exposure to altered metabolic conditions, resulting in non-alcoholic fatty liver disease (NAFLD); ii) chronic infection by hepatitis B virus (HBV) and hepatitis C virus (HCV); iii) chronic ethanol consumption; iv) persisting autoimmune injury either towards hepatocytes (autoimmune hepatitis type I and II) or cholangiocytes (primary biliary cholangitis or PBC, primary sclerosing cholangitis or PSC); v) genetically-related disorders (hereditary hemochromatosis, Wilson's disease, α-1-anti-trypsin deficiency, genetic cholangiopathies) [1-6]. CLD progression proceeds through a long-standing sequence of chronic parenchymal injury and persistent activation of hepatic inflammatory response that, in turn, fuels chronic activation of liver fibrogenesis and wound healing response. CLD progression is a critical and clinically relevant issue, potentially reversible, resulting in parenchymal changes slowly (on average requiring at least 15-20 years) developing to liver cirrhosis (i.e., an advanced stage of CLD characterized by regenerative nodules of parenchyma delimited by fibrotic septa, and associated with significant changes in organ vascular architecture) and the occurrence of portal hypertension and related complications (variceal bleeding, ascites, hepatic encephalopathy and hepatorenal syndrome), eventually resulting in organ failure and/or development of hepatocellular carcinoma [3-9].

Irrespective of the specific etiology, liver fibrogenesis operates through a complex scenario involving a plethora of molecular mediators and signals as well as of cellular mechanisms and responses [1-10]. CLD progression is mainly sustained by the persistent activation of the two critical and heterogenous population of hepatic myofibroblasts (MFs) and macrophages (either resident or recruited), although other cells may modulate CLD progression, including T- and B-lymphocytes, natural killer (NK) and NK-T cells, progenitor cells and activated or damaged cholangiocytes [3-9]. In the scenario of a progressive CLD multiple pro-fibrogenic mechanisms are involved that are either common to CLD of different etiology or more specific and etiology-dependent [3-6,8].

In the present review we will first offer a synthetic and updated overview of major basic concepts in relation to the role of MFs and macrophages as well as of selected established and

emerging issues and mechanisms favoring or promoting CLD progression, with a focus on the emerging role of genetic and epigenetic factors as well as hypoxia, hypoxia-inducible factors (HIFs) and related mediators. Although any basic achievement may potentially represent a basis for designing novel therapeutic strategies, we will not here analyze the complex and rapidly moving field of targeted therapeutic strategies that have been designed to interfere with- and inhibit CLD progression, exhaustively reviewed elsewhere [3-6,9,10].

1.2 Hepatic myofibroblasts in the pro-fibrogenic scenario

Hepatic MFs mainly originate from activated hepatic stellate cells (HSC) [6,8,11] or, to a less extent, from portal fibroblasts; a minority of MFs may originate also from bone-marrow-derived precursors, mesothelial cells or, still controversial, through a process of epithelial to mesenchymal transition [3,12-15]. Hepatic MFs contribute to fibrogenesis and CLD progression by well characterized pro-fibrogenic responses [6,8,10], including: i) increased synthesis of ECM components and altered remodeling of ECM, mainly in response to transforming growth factor β1 (TGFβ1), reactive oxygen species (ROS) and other oxidative stress – related mediators as well as several other growth factors and cytokines; ii) proliferation and survival, mainly in response to platelet-derived growth factor (PDGF) or other mediators like basic fibroblast growth factor (bFGF), connective tissue growth factor (CTGF) and leptin, with survival being also sustained by TGFβ1; iii) pro-inflammatory role through the ability of MFs to produce and release chemokines like C-C-chemokine ligand -2 (CCL2) and -21 (CCL21) as well as interleukin-1β (IL-1β), with MFs suggested also to modulate the response of adaptive immune cells [1-10]; iv) the ability to migrate in a redox-dependent way and then to align to nascent fibrotic septa in response to several peptide mediators (PDGF, CCL2, vascular endothelial growth factor-A or VEGF-A, angiopoietin-1, Oncostatin M), intracellular ROS generation and hypoxic conditions [3,10,16-18]; v) a pro-angiogenic role, by releasing VEGF-A, angiopoietin-1, hedgehog ligands, PDGF as well as by expressing related receptors, being involved in the association of pathological angiogenesis and fibrogenesis observed in CLD [7,19]; vi) the ability to perpetuate the chronic fibrogenic scenario by releasing (autocrine/paracrine loops), major mediators like TGFβ1, PDGF, CCL2, VEGF, endothelin-1 (ET-1) [1-10].

In the last two decades several studies have outlined a scenario in which sustained activation of MFs derived from activated HSC (i.e., HSC/MFs), results from a complex dysregulation of multiple molecular pathways and mechanisms [9,20]. As summarized in Figure 1, almost all

hepatic cell populations involved in the pro-fibrogenic scenario, including the recently described mucosal-associated Invariant T (MAIT) cells in autoimmune diseases [21], can release mediators sustaining the activation of precursor cells into MF-like cells positive for α-smooth muscle actin (α-SMA). Most of these mediators are peptide ligands operating through the interaction with their cognate receptors to contribute to persistent activation of either HSC (Figure 2) or portal fibroblasts [3-9,12,20]. Only few cells have been described to release mediators able to down-regulate activation of MFs (pro-resolving macrophages) or to contribute to their selective killing (NK and NK-T cells). In addition, one should mention the pro-fibrogenic role of ROS released by damaged hepatocytes or activated kupffer cells, of damage-associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs) acting on toll-like receptors (TLRs) expressed by MFs, and the opposite action of adipokines like leptin (pro-fibrogenic) and adiponectin (anti-fibrogenic). Molecular dysregulation and MF activation are further stimulated by nuclear receptor signaling pathways or positively or negatively modulated by alternative sets of transcription factors or miRNAs (see Figure 2), as recently reviewed [9,20].

### 1.3 The role of macrophages in the pro-fibrogenic scenario

Hepatic macrophages play critical roles in either the progression or regression of CLD of different etiology, as extensively reviewed elsewhere [3,5,6-8,10,22]. Recent experimental and human studies have introduced several new concepts that have deeply modified old dogmas, making outdated also for this organ the traditional distinction between polarization into inflammatory (M1) and resolutive (M2) macrophages [23,24]. Major concepts concerning macrophages and CLD progression can be briefly summarized as follows: i) Kupffer cells (KC) and monocyte-derived macrophages (MDM) can adapt their polarization in response to different signals and mediators; these macrophages may undergo a spectrum of activation states and functions to either sustain injury resolution or contribute to CLD progression; ii) although both KC and MDM retain macrophages functions (phagocytosis, danger signal recognition, release of cytokines, antigen processing, the ability to orchestrate immune responses), KC have mostly a "sentinel function" whereas the role of MDM prevails in conditions of acute and, particularly, chronic liver injury [24]; iii) liver macrophages can express receptors able to recognize the alarmin high-mobility group box 1 (HMGB1), released by stressed/injured hepatocytes and other activated cells, including the receptor for advanced glycation end-products (RAGE) as well as TLR2 and TLR4; activation of HMGB1 receptors is emerging as critical in eliciting inflammatory responses in NAFLD

and ALD (including expression of proinflammatory cytokines and recruitment of immune cells to the site of liver injury); moreover, HMGB1 has been also reported to activate HSC and stimulate phenotypic responses of liver MFs [25]; iv) circulating MDM mostly derive from a chemokine (C-X3-C motif ) receptor 1 $(CX3CR1)^+$ $CD117^+$ lineage-negative ($Lin^-$) bone marrow progenitor; MDM can rapidly accumulate in injured parenchyma, adapt towards several phenotypes and modulate the behavior of other cell types, including MFs [24,26]; iv) MDM consists of two populations expressing high or low levels of lymphocyte antigen 6 complex, locus C1 (Ly6C) [24]; following injury and TLR interactions with DAMPs or PAMPs KC and HSC can recruit, mainly through the release of chemokine (C-C motif ) ligand 2 (CCL2) and chemokine (C-X-C motif ) ligand 1 (CXCL1), $CCR2^+$/Ly-$6C^{hi}$ monocytes into injured liver that develop into pro-inflammatory, pro-angiogenic and pro-fibrogenic Ly-$6C^{hi}$ macrophages able to sustain activation (and survival) of HSC and other precursor cells into MFs [7,23,24,27]; v) following the withdrawal of the etiological cause (involving a decrease in DAMPS/PAMPS, phagocytosis of cell debris and autophagy), Ly-$6C^{hi}$ macrophages can shift into Ly-$6C^{low}$ restorative macrophages, releasing anti-inflammatory cytokines (IL-10, IL-1Ra), regenerative growth factors (HGF, VEGF) and matrix degrading metalloproteinase (MMP) expression (MMP9, MMP12 and MMP13), to promote degradation of excess ECM components [22-24]. The role of Ly-$6C^{low}$ restorative macrophages is relevant in the context of fibrosis regression or resolution, providing that the cause of liver injury is removed. If the etiological cause is removed at an early stage of fibrosis, a resolution and a full recovery of liver architecture and function, is possible. In the presence of a more advanced stage of fibrosis or in cirrhotic livers partial recovery of the organ architecture and function (incomplete resolution) can still be achieved [22]. In the scenario of fibrosis resolution, Ly-$6C^{low}$ restorative macrophages can prevail, and is accompanied by a deactivation of MFs. During fibrosis regression/resolution HSC/MFs are removed either by undergoing apoptosis or through phenotypic reversion to quiescent HSC. Ly-$6C^{low}$ restorative macrophages can also contribute to fibrosis resolution by increasing apoptosis of HSC/MFs. Also NK cells can induce interferon-γ (IFNγ)-mediated apoptosis of activated HSC [28,29] and may contribute to fibrosis resolution/regression by clearing senescent HSC/MF [30]. In theory, also NKT cells may act as pro-apoptotic but this is more controversial from available literature data [31].

## 2. Established and emerging etiology-related issues and mechanisms in liver fibrogenesis

In the complex profibrogenic scenario described [3-8,22,23] and in addition to chronic activation of wound healing and MFs in CLD, a number of selected pro-fibrogenic issues and mechanisms may be considered as common or etiology-independent, including: i) the established role of oxidative stress and ROS; ii) the established role of ECM qualitative and quantitative changes; iii) the emerging roles of extracellular vesicles (EVs), iv) autophagy and v) hypoxia and hypoxia-inducible factors (HIFs), with HIFs-related mediators being also involved in vi) pathological angiogenesis, which is strictly associated to the development of fibrogenesis. The two latter points will be discussed later in this review. We will also briefly discuss selected additional issues that should be more considered as etiology-related [3]. The identification and characterization of etiology-dependent or -independent mechanisms, pathways and mediators has emerged as critical in designing and testing novel therapeutic approaches to counteract CLD progression (recently reviewed in ref. [3]).

### 2.1 The role of oxidative stress

Oxidative stress, defined as an imbalance between excess generation of ROS and other redox-related reactive intermediates (RedoxRI) and the ability of cells and tissues to inactivate them, occurs in any CLD [1-6,8,10,32-34] as a consequence of at least three events: i) the persistent impact of a specific etiology on parenchymal cells (i.e., ethanol metabolism in ALD, dismetabolic conditions and lipotoxicity in NAFLD, HBV and HCV infection, etc), leading to an increased generation of ROS by injured hepatocytes; ii) the release of ROS by activated innate immunity cells, mainly macrophages (resident or recruited); iii) the decrease in antioxidant defenses reported in almost any progressive CLD [32-34]. ROS and other RedoxRI can exacerbate hepatocyte cell death, then contributing to chronic activation of inflammatory response, but can also modulate, by affecting defined signal transduction pathways and/or transcription factors, the phenotypic responses of MFs; this includes oriented migration and increased expression of pro-fibrogenic (procollagen type I, tissue inhibitor of metalloproteases 1 or TIMP-1) and/or pro-inflammatory (CCL2) genes [1-3,10,17,34]. Although ROS may be generated through a number of sources, including selected cytochrome P450 isoforms, lysyl oxidase and mitochondrial respiration [33,34], a relevant role has been attributed to intracellular ROS levels generated within MFs as a consequence of activation of NADPH-oxidase (NOX) isoforms following the interaction of peptide ligands to their cognate receptors (including PDGF, TGFβ1, VEGF-A, bFGF, endothelin-1 or ET-1,

Ang-II, IL-1β, TNF, LPS and IFN-γ). [2,4-6,34-36]. HSC/MFs express the phagocytic NOX2 isoforms (which is expressed by macrophages and other cells of innate immunity) as well as the non-phagocytic NOX1 and NOX4 isoforms. Unfortunately, although often effective in experimental models, neither the use of specific inhibitors of NOX isoforms or of antioxidant molecules has been reported to exert an anti-fibrotic effect in humans [37,38].

2.2 The role of qualitative and quantitative changes in ECM

Fibrogenic progression of CLD involves excess deposition, qualitative changes and altered topographic distribution of ECM components that occur in parallel with inefficient tissue remodeling and increased expression (i.e., mainly by activated MFs) of TIMPs [7,22,39]. ALD- and NAFLD-related early fibrosis are typically characterized by the replacement of the ECM of the space of Disse, normally containing mainly collagen type IV and VI, with a fibrillary-like ECM composed mainly by collagen type I and III as well as fibronectin. This is relevant, since an altered ECM composition can significantly deregulate cell responses and signaling pathways, as recently reviewed [40], and lead to the so-called capillarization of liver sinusoids [1,3-6]. The occurrence of qualitative and quantitative changes in ECM composition and the formation of fibrotic septa can also favor tissue hypoxia (see later). Liver hypoxia is a relevant stimulus for the induction of pathologic angiogenesis that accompanies fibrogenesis, eventually resulting in vascular changes, the raise of portal hypertension and of related complications. In all these issues an additional critical role is played by endothelial dysfunction due to altered balance between vasodilators and vasoconstrictors [41,42].

2.3 The increasing relevance of extracellular vesicles (EVs) in CLD progression

The term extracellular vesicles (EVs) indicates an heterogenous population of small membranous structures that are released by cells of different nature in the surrounding tissue microenvironment or in the blood circulation [43-46]. These EVs have an increasingly appreciated role in both physiological and disease conditions, including cancer. They can shuttle a long range of signals from parental cells, including mRNA, non-coding RNAs (microRNA or miRNAs, long RNA mitochondrial associated tRNA, small nuclear and nucleolar RNA), DNA, proteins and lipids to several target cells. This can occur either in an autocrine or paracrine way (EVs released in the microenvironment) or in an endocrine way (EVs released in the circulation). EVs are classified in relation to their biogenesis as follows [43-46]: i) exosomes (approx. 40–150 nm in size), generated in multivesicular body of parental cells, that derive from early endosomes formed by the

endocytosis of the plasma membrane; exosomes mainly contain proteins, non-coding RNA (miRNA, long-RNA) and RNA interfering; typical markers of exosomes are CD63, synthenin-1, tumor susceptibility gene 101 or TSG101 and different integrins; ii) microvesicles, also referred to as microparticles or ectosomes (approx. 40-1000 nm in size), released by parental cells through direct budding of their plasma membrane; these EVs carry lipids, proteins, non-coding RNA and mRNA and can induce a variety of cell responses when internalized by target cells; typical markers of microvesicles are phosphatidylserine, actinin-4 and mitofilin; iii) apoptotic bodies, which are produced by cells undergoing apoptosis, with a more heterogenous size (approx. 100–5000 nm) and morphology.

EVs, particularly exosomes and microvesicles, have been recently implicated in the progression of CLDs and there is current interest on their use as diagnostic and prognostic biomarkers (the so called “EVs profiling” in a scenario of liquid biopsy), futurable putative targets and potent tools for therapeutic delivery. Several reviews have been published in recent years on the role of EVs in CLDs of different etiology [47-52] and here we will just recall some of the most relevant data and concepts related to fibrogenic CLD progression.

EVs can be released “in vivo” by hepatocytes, cholangiocytes, hepatic stellate cells and MFs, sinusoidal endothelial cells, KC, MDM and other immune-related cells [47-52]. Hepatocytes release mainly exosomes and microvesicles containing variable amount and type of nucleic acids or different proteins (apolipoproteins, coagulation-related proteins, cytosolic proteins and proteins involved in endosomal pathway or detoxification). Similarly, in CLD all innate and adaptive immune cells can release EVs [53], then providing a form of autocrine/paracrine communication between immune cells or between immune cells and other hepatic cell populations [47-52]. Also quiescent HSC can release EVs, shuttling between quiescent and activated HSC, that contain twist-related protein 1 (TWIST1) and drives miR‑214 expression, resulting in decreased mRNA levels of connective tissue growth factor (CTGF) and in the modulation of liver fibrosis [54]. Whatever the cellular origin, EV composition may vary in relation to the presence or absence of specific stressing and/or disease condition as well as in relation to the state of stimulation or differentiation [47-51]. As recently reviewed in relation to NAFLD, ALD, HCV and cholangiopathies [51,52], EVs profiling can be then useful for diagnosis of nonmalignant liver diseases and can even serve as diagnostic biomarkers for liver tumors like hepatocellular carcinoma (HCC) and cholangiocarcinoma. In a human study EVs from different immune cells

were differentially enriched in NAFLD ($CD14^+$ and $iNKT^+$ EVs) and chronic hepatitis C (CHC) patients ($CD4^+$ and $CD8^+$ MVs), allowing to reliably distinguish between NAFLD and CHC patients [55].

The liver is a very active site of EVs uptake and hepatocytes and macrophages internalize EVs via endocytosis (phagocytosis, micropinocytosis, caveolin-mediated and lipid-raft-mediated) [56-58]. The uptake of EVs by target cells is facilitated by the presence on these vescicles of tetraspannins, integrins, major histocompatibility complex (MHC)-I and MHC-II molecules, immunoglobulins, peptidoglycans and lectins. Concerning the role of EVs in fibrogenic CLD progression [47-52] a number of studies and concepts deserve to be mentioned.

Most relevant data are related to EVs released by hepatocytes and at least the following major issues should be mentioned: i) EVs released by hepatocytes can induce in vitro dose-dependent proliferation of hepatocytes through transfer of sphingosine kinase 2 and increase in target cells of sphingosine-1-phosphate [59]; ii) HCV chronic patients have high levels of circulating EVs containing single or double strand HCV RNA; moreover, human hepatocytes and HCV-infected HuH2 cells release EVs containing HCV RNA complexed with miR-122, argonaute 2 and heat shock protein 90 (HSP90), suggesting that EVs can transfer HCV infection and trigger viral replication in not-infected hepatocytes [60]; in addition, EVs released by HCV infected hepatocytes can also induce M2 polarization in macrophages to promote HSC activation [61]; iii) in NAFLD conditions, as a consequence of lipotoxicity, steatotic hepatocytes can release EVs exerting pro-angiogenic, pro-inflammatory and pro-fibrogenic actions, the latter likely by directly affecting HSC/MFs [62-65]; the pro-inflammatory action on macrophages may occur through death receptor 5 – dependent signalling pathway [66] but EVs released from fatty hepatocytes also activate NLRP3 inflammasome and increase IL-1β release following internalization by either macrophages or cells of hepatocellular origin [67]; iv) excess alcohol consumption has been reported to increase circulating EVs containing high levels of miRNA-122, miRNA-192 and miRNA-309; EVs containing miRNA-122 can be released by primary human and murine hepatocytes exposed to ethanol and taken up by macrophages that, in turn, show functional changes and sensitization to lipopolysaccharide (LPS)-induced proinflammatory responses [68,69]; v) EVs released by steatotic hepatocytes can also promote endothelial inflammation through delivery of miRNA-1, up-regulation of nuclear factor-kB (NF-kB) pathway and down-regulation of Kruppel-like factor 4 (KLF4) expression; this study, in addition to confirm their pro-inflammatory action, indicate that EVs released from steatotic hepatocytes can facilitate atherogenesis, suggesting a

novel mechanism underlying the clinical evidence of a close link between NAFLD and cardiovascular diseases [70].

2.4 The role of autophagy and endoplasmic reticulum (ER) stress

A series of experimental and clinical studies has delineated an interconnected and pro-fibrogenic role for autophagy and ER stress. Autophagy is a fundamental process that is involved in maintaining cellular homeostasis under physiological conditions and regulates cellular adaptive responses in the presence of cellular stress [71,72]. In CLD autophagy has emerged as a process that, by generating fatty acids from the cleavage of retinyl esters contained in the HSC lipid droplets, can elicit HSC activation into MF-like cells. The pro-fibrogenic role of autophagy has been confirmed in murine models by using mice deficient for autophagy-related protein 7 (ATG7), a critical autophagy regulator, and in cultured HSC/MFs treated with different inhibitors of autophagy or knocked down for Atg7 or Atg5 expression [73,74]. In further studies a link between ER stress (that signals for expression of pro-fibrogenic genes in activated HSC), increased autophagy and activation of HSC/MFs was characterized. The block of the inositol-requiring enzyme 1α (IRE1α) pathway of the ER stress – related unfolded protein response (UPR) was sufficient to inhibit in a p38 mitogen-activated protein kinase (MAPK)-dependent manner fibrogenic response, resulting in down-regulation of HSC activation and autophagy [75]. Similarly, experimental fibrosis was inhibited through HSC targeted lentiviral delivery of the ER stress marker protein 78 kDa glucose-regulated protein (GRP78) [76]. More recently, the involvement of IRE1α pathway was related to the role of X‑box binding protein 1 (XBP1), a transcription factor operating downstream of IRE1α and able to induce type I collagen expression in HSCs (inhibited by knocking down ATG7) [77]. Moreover, ER stress has been proposed to promote fibrogenesis through dysregulation of miR-18A which is mediated by activation of PKR-like endoplasmic reticulum kinase (PERK) and consequent destabilization of heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) [76].

Other connections between fibrogenesis, autophagy and ER stress involve the collagen-specific molecular chaperone heat shock protein 47 (HSP47), critical for type I collagen maturation and secretion, and c-Jun N-terminal kinase (JNK) signaling. When HSP47 is depleted in activated HSC they accumulate immature and partially aggregated type I procollagen; this event was significantly increased when autophagy was inhibited, then up-regulating the expression of ER stress-inducible proteins like BiP (immunoglobulin heavy chain-binding protein) and Grp94 (94-kDa

glucose-regulated protein) [78]. Concerning JNK involvement, JNK signaling occurs downstream of ER stress and is believed to be involved in hepatocyte death, steatohepatitis and fibrogenesis [79]. Accordingly, JNK signaling in HSC/MFs, but not in hepatocytes, is critically and specifically contributing to pro-fibrogenic phenotypic responses as shown in murine models of CLD by using either JNK inhibition or JNK-1 deficient mice or in cultured cells [80,81].

Finally, in a recent study it has been reported that autophagy is a critical process able to maintain homeostasis of liver sinusoidal endothelial cells (SEC) being up-regulated during sinusoidal capillarization. This study suggests that downregulation of endothelial autophagy during chronic liver injury results in SEC dysfunction and induce in these cells an impaired ability to handle oxidative stress, overall exacerbating fibrosis [82].

## 3. Selected critical and emerging etiology-dependent issues and mechanisms in fibrogenesis

### 3.1 ECM deposition and histopathological patterns of fibrosis development

As recently reviewed, the specific etiology has a critical impact in the modulation of CLD progression [3]. Indeed, as originally proposed several years ago [83] and subsequently refined [3,84,85], the etiology can lead to at least three different histopathological patterns of fibrosis (Figure 3) also in relation of the prevailing profibrogenic mechanism(s) or the origin of MFs: i) the pattern of post-necrotic or bridging fibrosis, typical of CLD due to chronic viral infection or autoimmune hepatitis; this pattern is characterized by the development of fibrotic septa that, following persisting hepatocytes death and chronic inflammatory response, bridge portal areas to central vein areas (i., porto-central septa) or adjacent portal areas (i.e., portal-portal septa); in these settings the formation of septa leads to a relatively early derangement of vascular connections with the portal system and portal hypertension development; chronic activation of wound healing response and oxidative stress prevail as profibrogenic mechanisms, with MFs originating mainly from activated HSC and, to a less extent, portal fibroblasts; ii) the pattern of perisinusoidal/pericellular fibrosis, typically observed in NAFLD patients (mainly obese individuals often exibiting type II diabetes in the setting of the metabolic syndrome) progressed to non-alcoholic steatohepatitis (NASH) or in ALD patients developing alcoholic-steatohepatitis (ASH); at least in the early phases of both NASH or ASH, MFs mostly originate from activated HSC and lead to excess deposition of ECM in the space of Disse (i.e., perisinusoidal fibrosis) or around groups of hepatocytes (i.e., pericellular fibrosis); although oxidative stress and ROS play again a significant pathogenic role, here the CLD scenario seems more complex and further relevant pro-fibrogenic

mechanisms are involved in the progression of NAFLD and ALD; iii) the pattern of biliary fibrosis, which is detected in PBC and PSC, following an immune-mediated attack to cholangiocytes, or other cholangiopathies either congenital (biliary atresia, congenital hepatic fibrosis) or due to miscellaneous causes (toxic, inflammatory, ischemic or infectious) [86]; the pattern of biliary fibrosis in almost all forms of cholangiopathies is related to chronic damage to cholangiocytes and involves the activation of either portal fibroblasts or HSC into MFs, resulting in excess deposition of ECM components in portal areas and MFs proliferation; these events are concomitant to the so-called ductular reaction, a term that encompass proliferation of reactive cholangiocytes or reactive ductular cells (RDC) that can derive from proliferation of pre-existing cholangiocytes but also from so-called ductular metaplasia of hepatocytes and/or from hepatic progenitor cells [86]; this pattern results in the expansion of the portal tract and later on in the formation of portal-portal septa, then delaying the onset of portal hypertension and other related complications. The only apparent exception to this scenario is represented by Alagille syndrome which is characterized by a pattern of pericellular fibrosis and is less fibrogenic than other cholangiopathies (for example biliary atresia or congenital hepatic fibrosis).

In the last decade the computer-aided image analysis of the so-called collagen proportionate area or CPA (i.e., the proportion of the tissue sections stained by using the picro-Sirius red histochemical procedure) has revealed that the etiology has an impact on the overall deposition of ECM components [87]. Using this procedure, ECM deposition was found to be higher in pre-transplant cirrhotic livers from ALD patients than from PBC or PSC patients, with the lower levels of ECM deposition detected in cirrhotic livers from patients with autoimmune hepatitis or chronic infection by HCV or HBV [87]. More recently, digital analysis of CPA in cirrhotic livers has been proposed as a procedure offering superior results in terms of sub-classification of cirrhosis and of prognosis [88] and suitable as independent predictor of long-term outcome in NAFLD patients [89]. In the next sections we will offer a synthetic overview of few selected emerging issues and mechanisms which seems of particular interest and are mainly related to NAFLD progression and to chronic cholangiopaties.

### 3.2 Emerging issues and mechanisms in NAFLD progression

NAFLD is rapidly emerging as the most common cause of CLD worldwide, with a global prevalence of 25 % in the general population and even higher among obese individuals and/or patients affected by Type II diabetes mellitus [90,91]. Approximately 20-30% of NAFLD patients

can develop NASH, characterized by parenchymal cell injury, persistent lobular inflammation and potentially progressing to fibrosis, cirrhosis and liver failure [92,93]. Moreover, NAFLD has no validated therapy at present and is rapidly becoming a major cause of HCC (i.e., the 2nd leading cause of cancer-related mortality worldwide and now increasingly diagnosed also in non-cirrhotic livers), with just a minority of HCC patients surviving at 5 years from diagnosis [94,95].

The previous considerations and the high prevalence of NAFLD in the general population, have stimulated intense pre-clinical and translational research that have outlined the following issues and mechanisms (in a continuously evolving scenario of "*multiple parallel hits*") that are likely to prevail in driving NAFLD progression: i) the role of lipotoxicity and other issues that can mediate hepatocyte injury; ii) the role of intestinal microbiome, gut dysbiosis and altered gut-liver axis [96-100]. An additional critical issue for both NAFLD and ALD progression relies on the emerging role of genetic and epigenetic factors [101] that will be analyzed later in a dedicated section.

3.2.1 Lipotoxicity and NAFLD progression

The genesis of fatty liver in NAFLD patients is the overall result of increased hepatic delivery and/or availability of free fatty acids (FFA), either released in excess by insulin-resistant (i.e., dysfunctional) adipose tissue, increasingly synthetized in the liver (i.e., de novo lipogenesis) and/or following excess income of dietary lipids [100]. A critical role is played by dysfunctional adipose tissue which is expanded and become hypoxic during weight gain in obese patients, leading to adipocyte death, recruitment of M1 macrophages and release of pro-inflammatory cytokines which mediate insulin resistance and related increased lipolysis [99,100]. However, fatty liver by itself (i.e., triglyceride (TG) accumulation), is a positive adaptative event preventing NAFLD progression and should not be considered as responsible of hepatocyte injury [99,100]; indeed, genetic manipulation in mice has revealed that blocking the synthesis of TG can prevent fatty liver but exacerbates liver injury and fibrosis in experimental NAFLD [102].

A central role in determining hepatocyte death in progressive NAFLD is played by lipotoxicity exerted by several harmful lipid molecules, including saturated fatty acid like palmitate and stearate, short chain fatty acids, lysophosphatidylcoline and ceramides. Lipotoxicity is also defined as a dysregulation of the lipid environment and/or intracellular lipid composition leading to hepatocellular accumulation of these toxic lipids eventually leading to hepatocyte injury and death (i.e., lipo-apoptosis) [99,100]. Lipotoxicity is intimately associated with chronic inflammation

(also referred as metabolically-triggered inflammation, or meta-inflammation) and operates through a number of effectors, including: i) persistent induction of ER stress and activation of the UPR response that, instead of be adaptive or defensive, can activate all the three major downstream mediators of UPR (IRE1α, PERK and activating transcription factor 6 or ATF6) and trigger apoptotic and inflammatory pathways, the latter through the mentioned release of EVs [65-68]; ER stress and UPR-related death are due to up-regulation of pro-apoptotic genes like CCAAT-enhancer-binding protein homologous protein (CHOP, a pro-apoptotic transcription factor) and persistent activation of JNK isoforms [102-104]; ii) persistent activation of JNK isoforms, elicited not only by ER stress but also by oxidative stress, leading to lipoapoptosis [104], that also mediates inflammatory response and polarization in macrophages [105]; JNK activation mediates lipoapoptosis through increased expression of p53 upregulated modulator of apoptosis (PUMA, in turn up-regulated through CHOP) and BCL-2-associated X protein (BAX) [106]; iii) downstream signaling to Trail receptor 2 or DR5, upregulated by the CHOP and by JNK during ER stress, and mediating lipotoxicity exerted by palmitic acid [107]; iv) defective induction of the autophagic flux in hepatocytes, particularly of lipophagy (i.e., potentially able to regulate lipid content), through the proposed action of Sirtuin 3 [108]; v) mitochondrial damage and increased intracellular levels of ROS and other related redoxRI, with oxidative stress being also related to up-regulation of cytochrome P450 2E1 (CYP2E1) in NAFLD conditions [109]. Before leaving this section one should recall that lipotoxicity is also the cause of the release of pro-fibrogenic and pro-angiogenic EVs from steatotic hepatocytes.

3.2.2 Intestinal – related issues in NAFLD progression

Gut microbiota defines the complex of microorganisms harboured by any individual which is characterized by a high number of genes that are collectively referred to as microbiome. In non-obese individuals almost 90% of the microbiota belong to the two phyla, of Firmicutes and Bacteroidetes, with other phyla (Actinobacteria and Verrucomicrobia) being usually less represented [110,111]. The analysis of the distal gut microbiota in both obese individuals and genetically obese mice (versus lean individuals and control mice) has revealed that obesity is associated with defined changes in the relative abundance of the two dominant bacterial phyla, with an obesity associated decrease in Bacteroidetes and an increase in Firmicutes [112]. Of relevance, qualitative and quantitative changes in gut microbiota (referred to as gut dysbiosis and

also leading altered gut barrier function) have been proposed as predisposing factor favoring the development and progression of several chronic diseases, including NAFLD [99,100,111-114].

The role of changes in gut microbiota in the progression of NAFLD and other CLDs involves a number of mechanisms, as recently reviewed [99,100,111-114]: i) a suppression of the production by enterocytes of fasting-induced adipocyte factor (Fiaf), also known as angiopoietin like 4 (ANGLPTL4), which is a lipoprotein lipase inhibitor; suppression of this factor has been linked to increased fat-deposition in both adipocytes and hepatocytes [114]; ii) the altered gut barrier function allows translocation of bacteria and bacterial products that can then bind to specific toll-like receptor (TLR) isoforms on hepatocytes and adipocytes to induce pro-inflammatory pathways [100,115,116]; iii) the production of end-products of bacterial metabolism in the colon such as short chain fatty acids (SCFAs) from digestion of a variety of fibers; some of these SCFAs have been reported to deeply regulate host metabolism [117], likely by operating as substrates for gluconeogenesis and de novo lipogenesis in the liver; iv) the alteration of bile acid (BAs) levels that, although metabolized by bacteria, can prevent in the gut bacteria overgrowth and maintain gut homeostasis [118]; in relation to NAFLD, BAs can regulate lipid and glucose metabolism upon binding to farnesoid-X-receptor (FXR) that are nuclear receptors present either in hepatocytes and enterocytes; activation of FXR in hepatocytes can affect pathways involved in fatty acid metabolism whereas activation of FXR in enterocytes, through their release of FGF15/19, can affect liver lipogenesis and gluconeogenesis [100].

## 4. Genetic and epigenetic factors in the progression of NAFLD, ALD and other CLD

### 4.1 Genetic variants in NAFLD and ALD progression

ALD and NAFLD can be considered as leading causes of CLD worldwide [90,91,119,120] and share common histopathological features that range from simple steatosis, steatohepatitis (ASH or NASH) and potentially progressing towards fibrosis, cirrhosis and HCC development. Of relevance, differences in disease severity and progression between NAFLD and ALD patients have been described with only a relatively small proportion of these patients (approx. 10-30%) progressing to steatohepatitis and to more advanced stages of disease [92,93,121,122]. Both diseases are complex and multifactorial and the risk for disease progression relies on a combination of environmental factors (alcohol consumption, sedentary lifestyle, inadequate diet, features of the metabolic syndrome and intestinal microbiota) and multiple genetic factors [123,124].

Epidemiological as well as familial and twin studies, indicate a genetic contribution to both ALD and NAFLD progression, as confirmed by genome-wide association studies (GWAS) that led to the identification of relevant phenotypes [101,123,124]. Most of critical genetic variants have emerged from studies involving NAFLD patients, experiencing or not disease progression and then in relation of disease severity, but at least three of these genetic variants have been also associated with ALD: i) patatin-like phospholipase domain containing-3 (PNPLA3) gene; ii) transmembrane 6 superfamily member 2 (TM6SF2) gene; iii) membrane bound O-acyltransferase domain containing 7-transmembrane channel-like 4 (MBOAT7) gene. These genetic variants and an additional one, concerning glucokinase regulator (GCKR) gene, have been strongly associated with NAFLD development and progression; not surprisingly, all four gene variants encode for proteins involved in the regulation of hepatic lipid metabolism [101,123,124].

The best characterized genetic variant is the so-defined common PNPLA3 non-synonymous variant (rs738409 c.444 C>G p.I148M), with the C>G mutation causing isoleucine to methionine change at the amino acid residue level (I148M). This loss-of-function mutation, that alters the structure of the active site, negatively affect the ability of the protein product to act as a lipase involved in lipid droplet remodeling [125], and also impairs very low density lipoprotein (VLDL) secretion [126], then favoring TG accumulation. This single nucleotide polymorphism was first proposed to increase TG accumulation and hepatic inflammation [127]. In subsequent studies, different laboratories associated PNPLA3 variant with NAFLD severity and progression (i.e., grade of steatohepatitis and severity of fibrosis) [128,129] and homologous results were also confirmed for progressive ALD [123,130]. Further studies have suggested that the PNPLA3 rs738409 variant was also associated to a significantly increased risk to develop HCC in NAFLD and ALD patients as well also in chronic HCV patients [131,132].

A second genetic variant is TM6SF2 (rs5854296 c.449 C>T, p.E167K). Although the exact pathophysiologic role of the gene product is still elusive, several studies (reviewed in ref. [123,124]) suggest its relationship with TG accumulation and plasma levels of lipoproteins, TG and cholesterol. Individual carrying the homozygous TM6SF2 E167K variant exhibit a higher risk of developing hepatic steatosis, NASH and advanced fibrosis but have reduced levels TG and of LDL [133-135]. By contrast, patients homozygous for the normal allele show increased secretion into the circulation of TG and cholesterol: these patients are then somewhat protected for liver disease but have a more pronounced dyslipidaemia and an increased risk to develop atherosclerosis

[136,137]. The TM6SF2 E167K variant has been also associated to an increased risk to develop ALD and to undergo progression of the disease [130].

A third genetic variant of interest is MBOAT7 (rs641738 C>T variant) [101,124]; the gene encodes for a protein involved in the remodeling of phosphatidylinositol with arachidonic acid in the Lands cycle. This variant is common and is linked to the 3' untranslated region of MBOAT7, being associated with downregulation of MBOAT7 expression and reduced levels of phosphatidyl-inositol containing arachidonic acid both in the circulation and hepatocytes [138,139]. This variant has been associated to increased severity of NAFLD [138] and to an increased risk to develop HCC [140] and later reported to be also relevant for ALD progression [130].

The fourth genetic variant associated with NAFLD progression is glucokinase receptor protein (GCKR) (rs780094 A>G variant) which is in strong linkage disequilibrium with another GCKR variant (rs1260326 C>T variant) that has been reported to inhibit the ability of GCKR to respond to fructose-6-phosphate. This results in the inability of glucokinase activity leading to increased glucose uptake in hepatocytes which, in turn, lead to increased levels of malonyl-CoA, promotion of lipogenesis and of fatty liver [141]; as for other genetic modifiers, also GCKR variants are associated to fibrosis in NAFLD patients [142]. PNPLA3, TM6SF2, and MBOAT7 variants have been also involved in the progression of other liver diseases associated with steatosis such as HCV-mediated CLD [101].

4.2 Other genes associated with NAFLD progression

Other genetic variants have been identified in case-control and cross-sectional studies that may have a role in NAFLD progression by regulating lipid metabolism, inflammatory response, insulin signaling, oxidative stress and fibrogenesis. This specific matter has been recently reviewed [101] and here just the most interesting gene variants will be offered.

Some gene variants, and their related protein products, have been reported to affect mitochondrial function. This is relevant since these organelles are known to operate in NAFLD to increase FFA oxidation that, although preventing TG accumulation, can result in increased intracellular levels of ROS [143]. Accordingly, literature has identified different gene variants that are associated with increased oxidative stress and increased NASH severity and fibrosis. The rs4880 C>T variant of the mitochondrial manganese-dependent superoxide dismutase (MnSOD) [144] is associated with decreased MnSOD mitochondrial targeting and import, leading to a reduction of antioxidant mitochondrial defenses. Interestingly, a decreased susceptibility to NASH

and fibrosis has been associated to the 866 G>A promoter region variant (rs695366) of the Uncoupling Protein 2 (UCP2); this mutation leads to increased expression of UCP2 and affecting its physiological role (UCP2 is regulating both mitochondrial redox status and energy dissipation by uncoupling oxidative phosphorylation and fatty acid export from mitochondria) [145].

Other gene variants linked to NASH and fibrosis severity have been described through the years (reviewed in ref. 101]); the following variants have recently proposed to play a role in inflammation and fibrogenesis: i) a genetic variant in interferon (IFN)-k3/IFN-k 4 region and, particularly, the IFNL3 locus rs12979860 CC genotype that leads to increased IFN-k3 production; this variant has been associated to an increased risk to develop lobular inflammation and severe fibrosis in NAFLD as well as HBV or HCV chronic patients [146]; ii) the wild type MER protocol-oncogene, tyrosine kinase (MERTK) is emerged as a kinase involved in modulation of response in immune cells as well as in the activation of hepatic stellate cells [147], a finding confirmed by the evidence that a non-coding variant of the gene, leading to reduced MERTK expression, protects against fibrosis in NAFLD patients and in patients with chronic HCV infection [148,149].

### 4.3 Epigenetic factors in NAFLD and ALD

Several studies proposed that epigenetic factors are involved in both development and progression of NAFLD and ALD; these factors can also reasonably interact with inherited risk factors to determine the individual susceptibility to these diseases [101,123,150]. Epigenetic changes can modify gene expression and phenotypic variation in the absence of DNA sequence changes, being also relatively stable and potentially transmittable to progeny. Epigenetic changes include changes in DNA methylation, changes in histone proteins and chromatin remodeling or RNA-based mechanisms involving non-coding RNAs. Concerning changes in DNA methylation, several experimental or clinical studies have outlined some critical issues affecting NAFLD severity and progression in the single individual, including the roles of intrauterine exposure to a high-fat diet, of ageing in derangement of mitochondrial energy metabolism as well as of fibrogenic signaling pathways (reviewed in ref. [101]). Concerning fibrosis severity, patients with progressive NAFLD or ALD exhibit changes in methylation of CpGs within genes known to affect fibrogenesis, including hypermethylation of peroxisome proliferator-activated receptor α and δ (*PPARα* and *PPARδ*, considered as anti-fibrogenic genes) and hypomethylation of classic pro-fibrogenic genes like *TGFβ1* and *PDGFα* [151]. Moreover, a human study has outlined that critical genes involved in

the methylation process, inflammation, and fibrogenesis all exhibit a stage dependent regulation, suggesting the involvement of epigenetic changes in CLD progression [152]. Of interest, the methylation has also been reported to regulate the expression of the PNPLA3 gene [153].

However, most of the available data on epigenetic factors for both NAFLD and ALD are related to the role of microRNAs (miRNAs) (reviewed in ref. [101,123,150]), which are short non-coding single-strands RNAs, 19–22 nucleotides long, that are able to regulate mRNA degradation or translation and then the expression of sets of genes and pathways [154]. Dysregulation of miRNAs has been proposed to have both prognostic and predictive value (miRNAs being stable in blood and in urine) not only for NAFLD and ALD but also in conditions of chronic viral infection, and overall liver fibrosis and HCC development [155], although the accuracy of circulating miRNA profiles still require standardization and independent cross-validation [156]. Whether ALD is concerned, the most convincing data are related to miRNAs that have been reported in humans or murine models to be either up-regulated (miR-155, miR-34a, miR-212 and miR-21) or down-regulated (miR-122). Interestingly, some of the same miRNAs, particularly mi-R34a, miR-122 and miR-21, have been reported to be dysregulated also in NAFLD patients. As recently reviewed [101,123,150,157], there are indications that any of these miRNAs plays a specific function in the CLD scenario.

Along these lines, miR-122, which is the most abundantly expressed hepatic miRNA, is down-regulated in the liver in both NAFLD and ALD-related conditions. MiR-122 has been involved in the induction of genes related to lipid metabolism, cholesterol homeostasis, cytokines and HIF1α as well as in the expression tight junction proteins. Accordingly, down-regulation of miR-122 has been suggested to be involved in steatosis, by favoring lipogenesis and impairing lipid secretion, as well as in fibrosis by up-regulating profibrogenic pathways in HSC/MFs (see Figure 2) [101]. In addition, miR-122 has been suggested also to be involved in favoring ALD-related altered gut permeability similarly to what also reported for miR-212 [157]. Up-regulation of miR-155 detected in experimental ALD has been related to a reduced hepatocyte expression of PPARα and increased expression of genes involved in lipid metabolism and uptake, including low-density lipoprotein receptor (LDLR), fatty acid binding protein 4 (FABP4) and Acetyl-CoA carboxylase 1 (ACC1) [158]. Similarly, miR-34a and miR-21 were reported to be also involved in the regulation of lipogenesis and lipid secretion in NAFLD patients [101]. Again concerning miR-155, its ethanol-related upregulation in macrophages decreased the expression of *PPARγ* but up-regulated that of

*TGFβ1* [158]. More data and details on the role of miRNAs in ALD and NAFLD development and progression can be found in recent reviews [101,123,150,157].

**5. Critical and emerging issues and mechanisms in cholangiopathies**

Cholangiopathies are a large group of chronic diseases affecting the human biliary epithelium that recognize different etiologies [159] and can be caused by: i) genetic causes, like in the case of Alagille syndrome, Caroli syndrome, ABCB4 deficiency, cystic fibrosis and polycystic liver disease; ii) immune-mediated causes, including the two most relevant diseases PBC and PSC as well as a number of more rare conditions like IgG4-associated cholangitis and autoimmune cholangitis, eosinophilic or mast cell cholangiopathy and graft versus host disease involving the liver; iii) unknown causes (i.e., idiopathic cholangiopathies), including biliary atresia and sarcoidosis; iv) infectious causes such as in recurrent pyogenic cholangitis, Cryptosporidium-associated cholangiopathy or in recurrent cholangitis in patients with a choledochoduodenostomy; v) malignancy, with cholangiocarcinoma being the most relevant one; vi) vascular causes (i.e., vascular cholangiopathies) including post liver transplant hepatic artery thrombosis and portal hypertensive biliopathy.

Similarly to other CLDs, cholangiopathies are typically characterized by a progressive clinical course that, irrespective of the etiology, share common pathophysiological mechanisms that include proliferation and apoptosis, cholestasis, inflammation, fibrogenesis, and eventually carcinogenesis [159-161]. Cholangiopathies are relevant since actually represent a significant indication for liver transplantation in adult patients (10-20% of cases) and, particularly, in pediatric patients (80% of cases). In these diseases the chronic injury to biliary epithelium can trigger a persistently activated pathological repair leading to the previously described pattern of biliary fibrosis (i.e., characterized by excess deposition of ECM in the portal areas and those surrounding the injured bile ducts) that can progress to biliary cirrhosis and then end-stage liver disease. Nevertheless, apart from the typical pattern of biliary fibrosis, these diseases retain some peculiarities that deserve to be mentioned that are intimately related to the peculiar role played in these diseases by reactive cholangiocytes, also referred to as RDC [162].

A recent and authoritative review [162] has updated actual knowledge concerning the role of cholangiocytes in both physiological and pathological conditions and here one can briefly mention the fact that these epithelial cells, which are lining intrahepatic and extrahepatic bile ducts, are heterogeneous in size and function and actively contribute to bile composition and bile

flow. However, according to the topic of the present review, the most interesting data and concepts are related to the contribution of RDC in CLDs, with a focus on their role in liver regeneration as well as in sustaining chronic inflammation and fibrogenesis.

Concerning liver regeneration, although the biliary tree can harbor the so called hepatic progenitor cells (HPCs) in the terminal ductules and the Canals of Hering, in physiological conditions and even under conditions of partial hepatectomy HPCs are not significantly involved and senescent/apoptotic or missing epithelial cells are usually replaced through self-replication of existing adult hepatocytes or cholangiocytes [163,164]. HPC are more significantly involved in a scenario of liver injury and repair; according to rodent's studies, HPC are involved following acute liver injury or in the frame of a progressive CLD, with HPCs being able in these conditions (i.e., in which hepatocyte proliferation is compromised) to expand and differentiate into both hepatocytes and cholangiocytes [165]. It should be noted, however, that in humans HPC are indeed activated in the vast majority of liver diseases even in the presence of minimal or modest degree of parenchymal injury in a way which correlates with the disease severity [166]. Apart from the fascinating scenario of signals and pathways that push HPCs in differentiation (see ref. [162] and references therein) proliferation of cholangiocytes occurs through an accelerated replication in response to a number of autocrine and paracrine mediators including growth factors (TGFβ, TNF), cytokines (IL-6) neuropeptides and hormones like testosterone and estrogens [162].

Under conditions of persistent injury to biliary epithelium cholangiocytes undergo activation in response to several known (infective, cholestatic, ischemic, toxic) and unknown insults; the reactive cholangiocytes or RDC are cells not only able to proliferate but also to express and release pro-inflammatory, pro-fibrotic and pro-angiogenic mediators in a scenario of intense cross-talk (orchestrated by a variety of autocrine and paracrine signals) with cells of innate and adaptive immunity and mesenchymal cells usually referred to as "ductular reaction" that, with the time, can lead to the pattern of biliary fibrosis [162,167]. Figure 4 offers a current view of the complex scenario of signalling and cellular crosstalk network during the course of biliary repair and fibrosis. The following are the major issues involved in chronic progressive cholangiopathies [159-162]: i) the *primum movens* in biliary repair and then fibrosis in this kind of diseases is always represented by biliary epithelial injury leading to a condition in which cholangiocytes undergo a derangement of the homeostatic equilibrium (i.e., injured/activated cholangiocytes) and/or undergo cell death (apoptotic or necroptotic); ii) cholangiocytes, even in normal conditions, have a peculiar immune-biological phenotype since they express Toll-like receptors (TLRs), that can

respond to PAMPs (including LPS) [162], as well as cytoplasmic pattern recognition receptors like NOD-, LRR- and pyrin domain-containing 3 (NLRP3); of interest, cholangiocyte TLRs and the NLRP3 inflammasome have been reported to play a role in almost all relevant cholangiopathies [168-170]; iii) signals from injured biliary epithelium (Figure 3) are sensed by all the cells involved in the ductular reaction, including cells of innate immunity (macrophages and neutrophils), portal fibroblasts and myofibroblasts, endothelial cells and, in some cholangiopathies, also lymphocytes, leading to an intense cross talk between all the involved cells; iv) activated cholangiocytes/RDC interact with macrophages and this results in recruitment of monocytes through CCL2, IL-1, CXCL10 and CXCL12, and overall drive macrophages to release mediators that concur to the pro-fibrogenic peribiliary environment [167,168]; v) signals from the inflamed bile ducts and RDC, in particular TGFβ1 and TGFβ2, IL-6, PDGF-B and CCL2, recruit and activate cells of mesenchymal origin, particularly portal fibroblasts and HSC that differentiate into MFs; in turn, MFs release soluble factors or EVs establishing an intense cross-talk with reactive cholangiocytes [51,160-162]; vi) again on the atypical phenotype of RDC (i.e., versus normal biliary cells) is the fact that in vivo they have been reported to express markers of EMT (down-regulation of E-cadherin, increased expression of vimentin, Snail and MMP-2), likely in a scenario of "partial EMT" allowing these cells to display a phenotypic plasticity (and motility ?) that may be useful in wound repair [171,172]; vii) the secretory profile of RDC, in particular the ability to secrete pro-inflammatory cytokines and then amplify pro-inflammatory and pro-fibrogenic response, has been proposed to be similar to the so-defined senescence-associated secretory response of senescent cells [173,174] and recently a first attempt to specifically target senescent RDC has been reported to ameliorate fibrosis in a genetic murine model of cholangiopathy [175]; viii) a final issue is the reported ability of cholangiocytes to establish interactions and cross-talk with T cells, that is potentially relevant in the scenario of PBC and PSC (which involve an autoimmune attack to biliary epithelium) but also of other cholangiopathies; early studies showed that cholangiocytes express HLA class I molecules and, upon activation, HLA class II molecules [176-177], although the role of these cells as antigen presenting cells was debated; more recently, cholangiocytes have been reported to present antigens to NKT cells (likely through interaction between CD1a and invariant T cell receptor or iTCR) and mucosa-associated invariant T (MAIT) cells (through interaction between MR1 and iTCR), resulting in activation of NKT and MAIT cells, with MAIT cells reported to release pro-inflammatory cytokines [178-179]; although we still do not know whether the recently proposed involvement of MAIT cells in autoimmune diseases [21,180] may be due to antigens presented by

cholangiocytes, it should be recalled that MAIT cells can also sustain pro-fibrogenic activation of HSC into MFs in these clinical settings [21]; apart from data on MAIT and NKT cells, the interaction between activated cholangiocytes and T cells is favored through protein expressed by RDC, like intercellular adhesion molecule 1 (ICAM1) [177] and vascular cell adhesion molecule 1 (VCAM1) [181] or chemokines released by RDC such as CXC-chemokine ligand 16 (CXCL16) and CCL20 [182,183]; these interactions have been proposed to recruit and to modulate T cells' activity and favoring their persistence at the site of injury.

**6. Hypoxia, hypoxia-inducible factors and related mediators in the fibrogenic progression of CLD**

In the last decade hypoxia and hypoxia-inducible factors (HIFs) as well as pathological angiogenesis have been implicated in the progression of CLD of different etiology, including development of hepatocellular carcinoma [2,3,10,184-186]. More recently, interest for hypoxia and HIFs is emerged in relation to progressive NAFLD in the scenario of metabolic diseases and obesity [187,188]. As for any tissue and organ, the terms hypoxia and normoxia are intrinsically related to the normal oxygen partial pressure ($pO_2$) to which the liver is exposed. Along these lines, the liver is unique in its vascular supply since it receives highly oxygenated blood via the hepatic artery and oxygen-depleted blood via the portal vein, with the blood flow directed towards the central vein generating an oxygen gradient in which $pO_2$ ranges from 60-65 mmHg (84-91 µmol/L) in periportal areas to 30-35 mmHg (42-49 µmol/L) in the perivenous areas [189]. This oxygen gradient is intimately related to the so-called liver zonation, a condition in which hepatocytes show distinct biochemical and functional heterogeneity across liver parenchyma. In normal liver, fenestrated SECs and then sinusoids allow exchange of nutrients, metabolites, and substrates between hepatocytes and blood. Liver zonation is also critical to support the capacity of liver parenchyma to regenerate, replace dead hepatocytes and restore its structure and function following injury [189]. Along these lines, molecular oxygen is a critical substrate for numerous biochemical reactions and for mitochondrial energy production as well as for several α-ketoglutarate (2-oxoglutarate)-dependent oxygenases involved in maintaining $O_2$ homeostasis. In addition, $pO_2$ is also related to the promotion of collagen maturation, has an impact on DNA and histone methylation status and concur to regulate nutrient- and energy-sensing pathways (mTOR, AMPK and autophagy), as well as the unfolded protein response (UPR) [188-191]. Whether the liver is specifically concerned, one should consider that an hypoxia response is not observed in physiological conditions but changes in $pO_2$, even relatively modest but able to elicit locally an

hypoxic response, are known to occur in the progression of CLD and during liver carcinogenesis [184-189]. In the following subsections we will recall only major concepts, issues and mechanisms that can have a role in fibrogenic CLD progression.

6.1 HIFs and the hypoxic response

HIFs are members of the basic helix–loop–helix Per–Arnt–Sim (bHLH-PAS) family of heterodimeric transcription factors composed by an α-subunit, oxygen sensitive, and a constitutively expressed β-subunit. Three isoforms of α-subunit have been described, including HIF1α (expressed in all cells and tissues) and HIF2α (expressed in a more limited number of cell types, including endothelial cells, hepatocytes, glial cells, myocardial cells, type II pneumocytes, duodenal and pancreatic interstitial cells) which are the two best characterized isoforms [190-193]; a third subunit, defined as HIF3α, is still elusive in functional terms and less characterized [194]. The heterodimer (HIF1, HIF2 or HIF3) is mostly formed through the binding of the α-subunit with HIF1β constitutive isoform, also known as aryl-hydrocarbon receptor (AhR) nuclear translocator (ARNT) (190-193), with HIFα subunits being able to form a heterodimer also with ARNT-2, an additional β-subunit expressed only in central nervous system and kidney [195]. Under normoxic conditions HIFα-subunits are regulated through two major mechanisms: i) they can be rapidly hydroxylated in their oxygen-sensitive domain by prolyl hydroxylases (PHD) and subsequently conjugated with a E3 ubiquitin ligase complex containing the von Hippel-Lindau (VHL) protein as a pre-requisite to finally undergo proteasome degradation [190-193]; ii) they can be also hydroxylated on an asparaginyl residue by the factor inhibiting HIF1 (FIH1) that prevents the binding of HIFα subunits with the transcriptional co-activator cAMP-response element binding protein (CREB)-binding protein (CBP) and histone acetyltransferase p300 (p300 HAT), then preventing the action as transcription factor [196]. In hypoxic conditions, HIFα-subunits are less hydroxylated or not hydroxylated (depending on the $pO_2$), can undergo stabilization and can form the HIF heterodimer and the transcriptional complex that includes the CBP/p300 co-activator. HIFs, by binding to the HRE (hypoxia-responsive elements) in promoter or enhancer sequences, can then increase the expression of numerous sets of target genes that are involved in the so-called hypoxic response [190-193]. This response include the up-regulations of genes that sustain shift towards anaerobic glycolysis (and the intimately related response to prevent intracellular acidification) or are involved in metabolism (and metabolic adaptation), proliferation, angiogenesis and vasodilation, inflammation, cell motility, survival, stemness and cell differentiation, to mention just the most critical and investigated ones.

One of the most interesting concept in relation to the transcriptional activity of HIFs, with a potential impact on CLD progression, is the knowledge that HIF1α and HIF2α, and then HIF1 and HIF2, may regulate the expression of common but also of distinct target genes [192,193]. Relevant examples of HIF1α and HIF2α common target genes are those encoding for the glucose transporter 1 (GLUT1), VEGF-A, carbonic anhydrase IX and XII, the anti-apoptotic protein Bcl2, the chemokine receptor CXCR4, the chemokine CXCL12, the IL-1β and the transcription factor Twist involved in EMT. Examples of HIF1α specific target genes are those encoding for glycolytic enzymes (including lactate dehydrogenase A, pyruvate dehydrogenase kinase 1 and phosphoglycerate kinase 1), for factors involved in autophagy (such as BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 or BNIP3 and the related ligand BNIP3L), for ECM remodeling enzymes (lisyl oxidase 2 or LOX2, procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 or PLOD2), for inducible NO synthase (iNOs) and for the inhibitor of mTOR signaling pathway REDD1 (regulated in development and DNA damage responses 1). Example of HIF2α specific target genes are those encoding for factors involved in cell cycle and proliferation such as cyclin D1 and transforming growth factor-α (TGFα), for antioxidant enzymes like superoxide dismutase 2 (SOD2) and catalase, for erythropoietin, for the ECM remodeling enzyme MMP-13, for the delta-like ligand 4 (DLL4) involved in Notch signaling, for the ancestral stemness factor Octamer 4 and for the M2 marker arginase 1.

An additional and critical issue, particularly in conditions of persistent liver injury and CLD progression, is that one should consider that HIFs can also operate their transcriptional activity in an oxygen-independent way. This statement indicates that HIF transcriptional activity can be elicited by a number of conditions or signals or mediators (largely involved in CLD) able to operate also independently on hypoxia that can be summarized as follows [2,197,198]: i) ROS, either generated in stressed conditions (i.e., in hepatocytes in CLD conditions) or as a consequence of ligand-receptor interaction leading to activation of NADPH-oxidase isoforms of following exposure to hypoxia, have been reported to inhibit PHD activity, then allowing HIFs to be stabilized and to operate; ii) activation of MAPK signaling pathways following interaction between peptide ligands and their cognate receptors can lead to phosphorylation and stabilization of HIFs, favoring their transcriptional activity; iii) HIFs have been reported to be stabilized in normoxic conditions following hepatocyte infection by HBV and HCV through still poorly characterized mechanism(s) (197).

### 6.2 Hypoxia, HIFs and pathological angiogenesis: a link to fibrogenesis

Fibrogenic progression of CLDs is known to be intimately associated with pathologic angiogenesis and sinusoidal remodeling. Pathological angiogenesis, which is a prototype of hypoxia- and HIFs- related response, has been extensively investigated in the past for a number of critical reasons: i) it has been unequivocally detected in all major conditions of CLD, irrespective of the etiology, and found to parallel fibrogenic progression (199,200); ii) in relation to the exposure to hypoxia and the HIF-mediated responses as well as to the intense interactions and mediators cross talk between hepatic cell populations (activated MFs, liver SECs, macrophages and hepatocytes), pathological angiogenesis can have a major role in sustaining and potentially driving liver fibrogenesis; iii) it has an established and widely accepted role in the genesis of portal hypertension and related complications; iv) several laboratories have investigated, initially in pre-clinical models with encouraging results, whether anti-angiogenic therapeutic strategies may be useful to prevent or slow down CLD fibrogenic progression (extensively reviewed in ref. [2,10,184-189,197,198,201]). At present, the interest in antiangiogenic therapies, which is still high in relation to liver carcinogenesis and hepatocellular carcinoma treatment, has been tempered in recent years by the substantial lack of efficacy of antiangiogenic drugs and related therapeutic strategies in human CLD patients [2,10,186,201].

If we go back to basic aspects, a number of critical established and emerging issues can be proposed that link angiogenesis, fibrogenesis and CLD progression, including the previously mentioned role of EVs in mediating a pro-angiogenic action. A first established issue is that hypoxic areas increase progressively in liver parenchyma from early injury to the development of cirrhosis. Accordingly, CLD progression by itself is contributing to self-perpetuation of hypoxia as a consequence of the impairment of oxygen diffusion which, in turn, is due to the formation of regenerative parenchymal nodules surrounded by fibrotic septa, to vascular remodeling and progressive capillarization of the sinusoids. This scenario can trigger a vicious circle in which parenchymal hypoxia, through HIF-mediated biological actions, up-regulates factors and mediators that persistently activate wound healing response. Since angiogenesis is inefficient in a progressive CLD, persistent tissue hypoxia and pathological angiogenesis can act synergistically in dysregulating normal tissue repair and then promoting fibrogenesis [186,188,199-201].

A second critical hypoxia-related issue relies on HIFs as factors able to sustain the interrelationships between inflammatory, angiogenic and fibrogenic response as well as, in particular, by modulating the interactions and the interrelated cross talk between MFs, macrophages and SECs. All these cells as well as hepatocytes can effectively respond to hypoxia

and HIFs and, as mentioned in previous sections, have a role in CLD progression and can potentially express and release factors involved in angiogenesis [186,188,199-201]. Most of available data are related to the pro-angiogenic role of activated HSC and more generally hepatic MFs. Indeed, MFs have emerged as cells able to both release and respond to pro-angiogenic mediators [186,188,199-201] although one should not forget the specific involvement of macrophages [202] and SECs [186,203] in relation to liver angiogenesis and CLDs.

Concerning the relationships between HIFs, macrophages and pathological angiogenesis, few data are currently available. It should be here at least mentioned that $CCR2^{+}$/Ly-$6C^{hi}$ monocytes (i.e., the prevailing phenotype of monocytes recruited in a CCL2-dependent way in chronically injured parenchyma) play an essential role in angiogenesis regulation since pharmacological CCL2 inhibition resulted in prevention of angiogenesis and angiogenesis-associated fibrosis but not of fibrosis progression [204]. As an indirect connection with hypoxic conditions, pro-inflammatory and pro-fibrogenic macrophages derived from $CCR2^{+}$/Ly-$6C^{hi}$ monocytes have been reported to co-localize with newly formed vessels in portal tracts and to express and release pro-angiogenic factors like VEGF-A and MMP9 expression [204].

The HIFs-related role of activated HSC and MFs is more complex and can be summarized as follows: i) MFs respond to hypoxia by HIF1α-dependent up-regulation of several factors, including VEGF-A, Angiopoietin-1 and their cognate receptors VEGFR2 and Tie2, selected chemokine receptors (CCR1, CCR5), interleukin-13 receptor-α1, prolyl-4-hydroxylase-α2 and placental growth factor [205-207]; ii) MFs respond to VEGF-A by enhancing proliferative response and synthesis of ECM components as well as by displaying oriented migration [16,17,186,207]; according to these first issues and in relation to the intimate interactions of activated HSCs (and likely MFs) and endothelial cells in chronically injured liver, it has been proposed that fibrogenesis and angiogenesis may be driven or modulated by both MFs and hypoxia, at least in an early phase in which neoangiogenic vessels are included in developing septa [186,205]; iii) a number of mediators have been reported to up-regulate, independently on hypoxic conditions, the pro-angiogenic role of activated HSC or HSC/MFs through either increased transcription of HIF1α or its increased stabilization (as described for conditions resulting in increased ROS intracellular levels); two examples of hypoxia-independent and HIFs-related mediators eliciting a proangiogenic response in HSC/MFs are represented by leptin and PDGF-BB; these two mediators, which are pro-angiogenic in vivo [208-210], can elicit a proangiogenic phenotype in cultured HSC/MFs through a common signaling pathway resulting in VEGF up-regulation and release that involves activation of

the mammalian target of rapamycin (mTOR) pathway and ROS generation through NADPH oxidase, the latter event being relevant for increased HIF1α stabilization [211].

A final mention in relation to angiogenesis and fibrogenesis should be dedicated to the role of the interactions established between liver SECs and HSC/MFs. It is now clear that under physiological conditions liver SECs can be considered as gatekeepers of liver homeostasis. SECs in normal liver display anti-inflammatory and anti-fibrogenic properties by preventing activation of kupffer cells and HSC, the latter also through the intimate contacts established with quiescent HSC (see ref. [203,212] for more details). Moreover, liver SECs also regulate intrahepatic vascular resistance and portal pressure. Under conditions of progressive CLD sinusoidal endothelium becomes dysfunctional (i.e., unable to generate vasodilator agents in response to increased shear stress) and undergoes capillarization (i.e., the loss of liver SEC fenestrae) with liver SECs starting to release pro-inflammatory and also pro-fibrogenic mediators, eventually failing in regulating HSC quiescence. Within this CLD scenario, liver SECs also release in a HIF1α-dependent way VEGF-A as well as angiopoietin 2 and closely interact with activated HSC to sustain pathologic angiogenesis in the chronically injured liver [186,203,212].

### 6.3 The role of HIF1α and HIF2α as well as of related mediators in CLD progression

In recent years the improvement of analytical techniques and the use of specific transgenic mice has allowed to increase our knowledge on the role of HIFs in the progression of CLD, although most of the available data are related to progressive NAFLD (resumed in Figure 5). This is related to the fact that, due to the efficacy of direct antiviral agents in contrasting viral infections (particularly in the case of direct antiviral agents used against HCV), progressive NAFLD is emerging as the most relevant CLD at least in western countries. As a consequence, a consistent amount of data is now available in relation to the role of HIFs in metabolic diseases and particularly for those conditions, like NAFLD, associated to obesity and type II diabetes [188]. The bulk of data obtained in this fascinating field has offered a number of relevant concepts including the fact that obesity can trigger hypoxia in adipose tissue and the small intestine, with HIF1α and HIF2 signaling potentially resulting in adverse metabolic effects like the development of insulin resistance and NAFLD itself [188]. Along these lines, following obesity-related hypoxia, HIF1α in adipocytes has a major role in inducing up-regulation of inflammatory mediators and downregulation of adiponectin expression, a scenario that results in the raise of insulin resistance in the adipose

tissue. HIF2α activation in the small intestine is, in turn, more related to the emerging role displayed by the intestine in potentiating obesity-associated metabolic diseases [188]. In the last two subsections we will just analyze a number of selected issues in relation to the specific role of HIF1α and HIF2α in CLD progression, with a major focus on NAFLD, and the interested reader can find a more exhaustive analysis of the role of HIFs in metabolic diseases in a recent review [188] and references therein. Before to start HIF1α- and HIF2α-dedicated subsections in CLD progression one has to mention that, differently from what reported for other diseases (such as atherosclerosis, adipose tissue inflammation and obesity, renal fibrosis, gastritis, arthritis as well as airway allergy and asthma, see ref. [213]) few data and studies have specifically analyzed the role of HIFs in relation to the modulation of the function of liver macrophages and other cells of innate immunity. Unfortunately, most of available data on HIFs have been obtained in relation to murine models of liver carcinogenesis and for human HCC [197,201,214].

### 6.3.1 HIF1α and CLD progression

As a general issue, it is believed that HIF1α may affect CLD progression as a consequence of its role (and of HIF1) in regulating the expression of genes involved in glucose and lipid metabolism, an issue that can assume a particular relevance in conditions of progressive NAFLD which are tightly related to obesity and type II diabetes [188]. Histopathological evidence indicates that hepatic hypoxia in NAFLD develops in parallel with fatty liver being detected mainly in peri-venous areas, similarly to what also reported for alcohol-induced steatosis [215,216]. Although both HIF1α and HIF2α have been reported to modulate the cellular adaptive responses to hypoxia, literature suggests that HIF1α mainly promote glucose consumption and glycolysis, while HIF2α seems to be mainly involved in regulating lipid storage (see next subsection) [190-193].

A first study implicating a direct role for HIFs in NAFLD was provided in a study employing mice carrying hepatocyte conditional deletion for VHL protein: in these mice, in which hepatocyte VHL disruption can result in an $O_2$-independent overexpression of both HIF1α and HIF2α, authors reported a rapid onset of liver steatosis and impaired fatty acid oxidation [217]. This effect was referred to a marked impairment of selected mitochondrial function and, particularly, to a marked suppression of mitochondrial respiration, that was prevented by simultaneous inactivation of HIF1β. Onset of fatty liver was also reported in mice carrying hepatocyte conditional deletion of PHD2 and/or PHD3, a genetic manipulation that can also lead to up-regulation of HIFα subunits [218]. Other Authors soon provided evidence indicating that, by using mice genetically

manipulated either to overexpress HIF1α or to carry its deletion, ethanol-mediated fatty liver is significantly exacerbated by HIF1α [219]. Authors also provided evidence that HIF1α activation in hepatocytes and lipid accumulation in ALD may be due, at least in part, to the action of the chemokine CCL2 on hepatocytes, then suggesting an interesting link between inflammation and fatty liver. Although HIF1α was confirmed to be up-regulated following exposure to ethanol, the role of HIF1α in relation to the onset of steatosis was challenged by a subsequent study, still performed in mice carrying hepatocyte-specific deletion of HIF1α: in this study HIF1α, in turn, was proposed as protective against ethanol-induced fatty liver [220].

HIF1α was also proposed as a factor being involved in the fibrogenic progression by a number of studies published from the same laboratory. The first of these studies provided data indicating that hepatocyte conditional deletion of HIF1α resulted in a significant reduction of liver fibrosis in the experimental rodent model of biliary fibrosis induced by bile duct ligation (BDL) [221]. Further experimental studies from the same group proposed a role for HIF1α in the up-regulation of profibrogenic responses in activated HSC [207]. Using myeloid cell-specific HIF1α or HIF-1β knockout mice and again the BDL experimental model, the same authors reported a decrease of pro-fibrogenic markers and of liver fibrosis and suggested that in liver macrophages HIF1α activation may promote fibrosis mostly by up-regulating PDGF-BB expression, a known mitogenic and chemotactic stimulus for activated HSC and MFs [222]. These data were corroborated by evidence for nuclear HIF-1α protein in macrophages, hepatocytes, and MFs in the livers of PBC and PSC patients [222]. Very recently, these Authors also suggested that both HIF-1α and HIF-2α may elicit CXCL12 expression in hepatocytes, a chemokine that is up-regulated in conditions of chronic liver injury and believed to be relevant for development of liver fibrosis [223]. A role for HIF1α in biliary fibrosis was also proposed by a preclinical study that employed the specific HIF1α inhibitor 3-(5-hydroxymethyl-2-furyl)-1-benzylindazole (YC-1) in the BDL model, resulting in a significant decrease of HIF1α as well as of liver fibrosis and angiogenesis [224]; interestingly, the effect of YC-1 was also related to a down-regulation of suppressor of cytokine signaling -1 (SOCS1) and -3 (SOCS3), inhibition of NF-kB activation and phosphorylation of signal transducer and activator of transcription (STAT)-3.

As mentioned in a previous section, it should be again stressed that HIF1α-dependent transcriptional program can be up-regulated in target cells not only by hypoxic conditions but also by a number of peptide mediators able to either increase HIF1α stabilization or transcription. In addition to the previously mentioned examples of leptin and PDGF-BB, Oncostatin M (OSM) has

been recently proposed as a HIF1α-related additional pro-fibrogenic mediators [18,225]. OSM, a cytokine belonging to the IL-6 family, is of particular interest because although produced and released by cells of innate immunity its receptors have been reported to be expressed also by hepatocytes and activated HSC [18,226,227]. The first report concerning OSM was obtained in hepatocytes and hepatoma cells where OSM signals through two distinct heterodimeric receptors containing gp130 and, alternatively, either leukemia inhibitor factor receptor β (LIFRβ) or OSM receptor β (OSMRβ). OSM in these cells was found to elicit HIF1α-related downstream signaling events [226] but was also proposed to affect the response of HSC/MFs by up-regulating collagen I and TIMP1 expression [227]. The pro-fibrogenic action exerted by OSM was confirmed by a mechanistic study in which this cytokine was shown to be able to up-regulate the expression of TGFβ1 and PDGF in macrophage, growth factors known to act on HSC/MFs [225]. Very recently, our group has provided data indicating that OSM can exert a direct action on human HSC/MFs by inducing oriented migration through a downstream signaling involving early intracellular ROS generation and activation of Ras/Erk, JNK1/2, phosphatidylinositol-3-kinase (PI3K) and STAT1/STAT3 but also by up-regulating a HIF1α-dependent increased expression and release of VEGFs [18]. Of interest, we also provided for the first time that OSM and OSMRβ were overexpressed in the liver of human NASH patients as well as in murine liver in mice fed on three different dietary protocols resulting in experimental progressive NAFLD [18].

### 6.3.2 HIF2α and CLD progression

As previously mentioned, HIF1α and HIF2α, from a transcriptional point of view, can trigger the expression of both common and specific gene targets. However, despite a quite consistent bulk of literature data exists on the role of both HIF1α and HIF2α in the carcinogenic process (including liver carcinogenesis) [185,197,214,228], the role of HIF2α in the fibrogenic progression of CLD has only recently been investigated and mostly in relations to progressive NAFLD. This is somewhat surprising since hypoxic conditions have been earlier reported to stimulate lipid storage and inhibit lipid catabolism through β-oxidation [187,191,229] and HIF2α was already proposed as a critical factor involved in lipid storage [192,229,230]. Indeed, some years ago two studies were performed on VHL and, alternatively, either HIF1α- or HIF2α-double-knockout mice, a strategy designed to stabilize just one of the two subunits. These early studies already showed that HIF2α,

and not HIF1α, was responsible for the development of severe hepatic steatosis [231,232]; one of these studies also showed that HIF2α was acting by up-regulating genes involved in fatty acid synthesis/uptake and lipid storage, as well as by down-regulating those involved in fatty acid catabolism [232]. In the latter study it was reported that HIF2α may be also related to an early increase in the transcription of inflammatory cytokines and then to the development of steatohepatitis [232] as well as in activation of pro-angiogenic mediator angiopoietin-related protein 3 (ANGPTL3) or even some pro-fibrogenic genes. However, both studies were performed on mice carrying multiple genetical manipulations in the absence of liver injury [231,232] or in the presence of a very short (i.e., two weeks) protocol of ethanol administration [232], then in conditions unlikely to reproduce what happens in human conditions of NAFLD or chronic ethanol consumption.

Only several years later a study provided more compelling evidence that hepatocyte HIF-2α activation was a key issue in both experimental and, for the first time, in human NAFLD [233]. In this study HIF-2α was selectively overexpressed in the nuclei of hepatocytes in a high percentage of biopsies obtained from NAFLD patients at different stages of the disease progression, and homologous data were obtained in murine NAFLD. Hepatocyte conditional deletion of HIF2α in mice fed on a dietary regimen for progressive NAFLD led to a decrease in parenchymal injury, steatosis, lobular inflammation and liver fibrosis [233]. In vivo and in vitro experiments outlined that HIF2α was acting by directly regulating the hepatocyte production of histidine-rich glycoprotein (HRGP). Studies on human specimens showed that HRGP was overexpressed in all patients showing HIF2α hepatocyte nuclear staining, with a significant positive correlation between HIF-2α and HRGP liver transcript levels in these patients [233]. This is relevant since HRGP, a hepatokine (i.e., a pro-inflammatory cytokine produced and released by stressed hepatocyte) up-regulated in CLD, was mechanistically shown to be relevant for the progression of both human NAFLD and HCV-related CLD as well as for murine CLD by potentiating macrophage M1 migration and polarization and then resulting in increased liver inflammation [234]. The HIF2α-dependent up-regulation of HRGP in hepatocyte, driving macrophages to M1 polarization, is of interest for at least two reasons: i) literature data usually suggest that HIF1α and HIF2α have opposite effects on macrophage polarization, with HIF1α being pro-inflammatory and driving macrophage M1 polarization whereas HIF2α is more anti-inflammatory and sustains M2 polarization (see ref. [188] and references therein); ii) recently, SerpinB3, another HIF2α-dependent mediator again released by stressed or hypoxic hepatocytes during the course of

human CLD [235,236], has been reported to act as an effective profibrogenic mediator either in vivo in transgenic mice overexpressing SerpinB3 in hepatocytes in conditions of chronic liver injury (induced by either a NAFLD-related dietary regimen or by chronic administration of hepatotoxin carbon tetrachloride) or in vitro by up-regulating pro-fibrogenic genes in activated HSC/MFs [237]. Moreover, in the same study evidence was provided that also the inflammatory response was increased in SerpinB3 transgenic mice and this issue is at present under investigation in our laboratory. The overall message emerging from these studies suggests that HIF2α may operate in a pro-inflammatory and pro-fibrogenic way by up-regulation of the expression of selected mediators by stressed hepatocyte in CLD progression, particularly in NAFLD.

An additional interesting issue, that is also a note of caution in designing strategies to block HIF2α, is that involvement of liver HIF2α activation has been described to ameliorate hyper-glycaemia through an insulin-dependent pathway, with increased levels of insulin receptor substrate 2 or through the insulin-independent pathway through repression of glucagon action [238-240]. As discussed in a recent review on this specific topic, pharmacological inhibition of hepatic HIF2α might not be optimal for NAFLD therapy owing to the risk of increased hepatic glucose production and to exacerbate diabetes type 2 [188]. On the other hand, the scenario of HIF2α involvement in metabolic diseases (then in obese and type 2 diabetes patients that are also often showing NAFLD) is much more complex since HIF2α has a role also in what happens at the level of adipose tissue and intestine [188]. In particular, it has been shown that in both obese humans and in experimental dietary induced obesity HIF2α activation was directly related not only to obesity but also to hepatic steatosis. Along these lines, the experimental use of the HIF2α specific inhibitor PT2385 resulted in intestine HIF-2α inhibition and improved both obesity and liver steatosis [241].

## 7. Conclusions

Liver fibrogenesis, as proposed in this review, is a dynamic and highly integrated molecular, tissue and cellular process resulting in the progressive accumulation of extracellular matrix (ECM) components, mainly fibrillary collagen. Liver fibrogenesis, together with persisting inflammatory response, is a major driving force for CLD progression, irrespective of the specific etiology. Hepatic MFs as well as cell of innate immunity, particularly activated macrophages (either resident or recruited from peripheral blood), have a major role in the slow and often

longstanding development of more advanced stage of disease, eventually leading to cirrhosis and related complications, liver failure and the development of hepatocellular carcinoma. In the present review we have offered an update on major established and emerging basic concepts, issues and mechanisms outlined in either preclinical as well as clinical studies. This bulk of knowledge will help us to progressively unravel molecular, cellular and tissue mechanisms critical for disease progression as well as to design novel and more specifically targeted therapeutic strategies to be employed as an alternative to liver transplantation (at present the only effective therapy for end-stage liver disease).

**Acknowledgements**

The research leading to these results has received funding from AIRC under IG 2017 ID. 20361 project – P.I. Parola Maurizio. This research was also partially funded by the University of Torino (Fondo di Ateneo ex 60% - SC, EN, MP).

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## Figures and captions

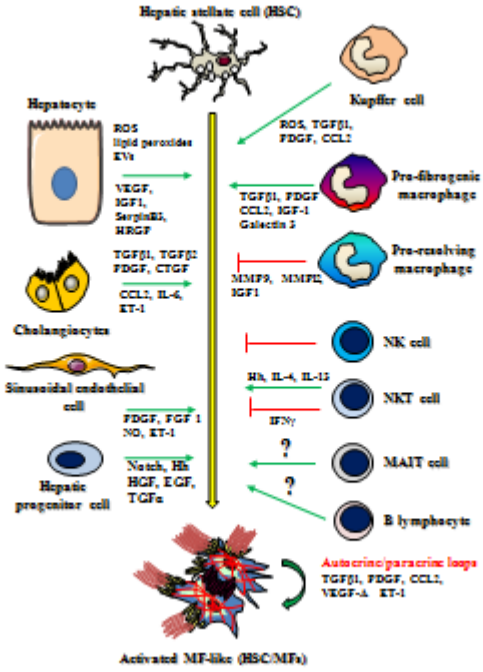


**Figure 1. Cellular interactions and mediators involved in the activation of HSC to MF-like phenotype.** The activation of HSC is modulated by several mediators or signaling pathways or other released by almost all cell populations involved in CLD, including hepatocytes, cholangiocytes, liver sinusoidal endothelial cells, hepatic progenitor cells, Kupffer cells, pro-fibrogenic and pro-resolving macrophages, natural killler (NK) and NLT cells, MAIT cells and B lymphocytes. Mediators can either trigger and sustain (green arrows) or inhibit (red lines) HSC activation. Activated HSC or HSC/MFs also release in autocrine/paracrine loops a number of mediators involved in self-perpetuating the activation. NK cells can kill early activated or senescent HSC similarly to what described for NKT cells in a IFNγ-dependent manner. The mechanisms or mediators involved in the action of MAIT cells or B lymphocytes are still unknown. Abbreviations: CCL, C–C motif chemokine ligand; CTGF, connective tissue growth factor; CXCL, C–X–C motif chemokine ligand; EGF, epidermal growth factor; ET1, endothelin 1; Evs, extracellular vesicles; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; Hh, hedgehog; HRGP, histidine-rich glycoprotein; IGF1, insulin-like growth factor 1; MMP, matrix metalloproteinase; NOS, nitric oxide synthase; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

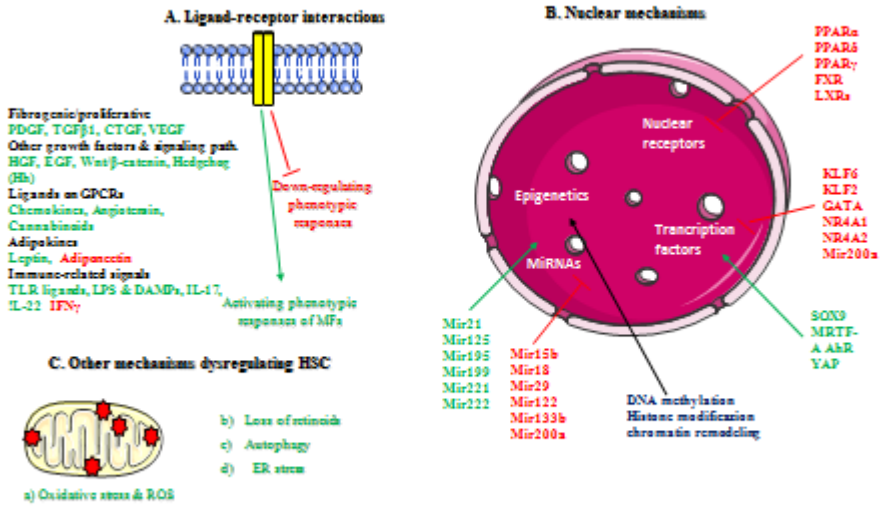


**Figure 2. Mechanisms, signaling molecule and pathways resulting in dysregulation and activation of HSC.** Phenotypic responses of activated HSC can be either triggered (in green) or inhibited (in red) as a consequence of: A) the action of mediators from the extracellular environment acting on their cognate receptor, including platelet-derived growth factor (PDGF); transforming growth factor-β (TGFβ), connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), Wnt/β catenin, Hedgehog (Hh) ligand, chemokines, angiotensin, cannabinoids, Toll-like receptors (TLRs) ligands, lipolysaccharide (LPS), damage-associated molecular patterns (DAMPs), interleukins (IL), interferon γ (IFNγ); B) nuclear mechanisms based on either the action of MiRNAs or transcription factors with opposite action or through the negative action of nuclear receptors; a role for epigenetic mechanisms is also emerging; C) a number of established mechanism able to trigger and/or sustain HSC activation into HSC/MFs such as oxidative stress and reactive oxygen species (ROS), loss of retinoids, autophagy or ER stress. Other abbreviations: KLF , Kruppel-like factor; FXR, farnesoid X receptor; LXR, liver X receptor; MiR, microRNA; NR4A1, nuclear receptor subfamily 4 group A member 1; PAR2, proteinase-activated receptor 2; PPARs, peroxisome proliferator-activated receptors; VDR, vitamin D3 receptor.

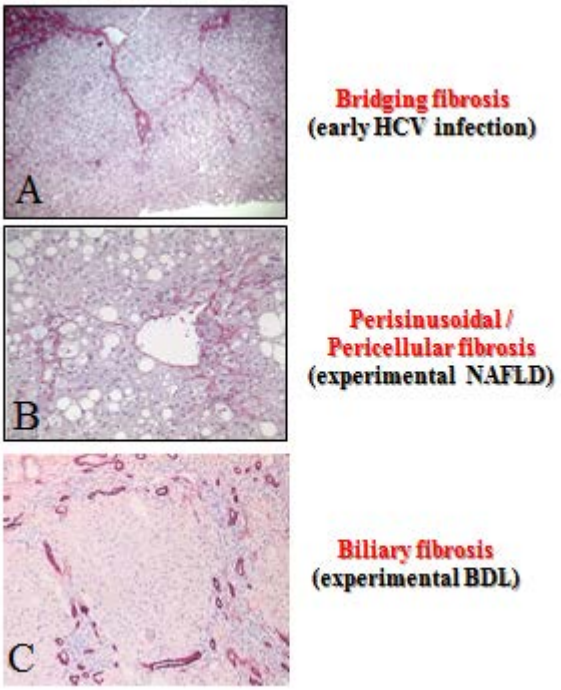


**Figure 3. Histopathological patterns of liver fibrosis. A.** Bridging fibrosis in a human biopsy from a patient with early HCV chronic liver disease, showing formation of septa bridging portal areas or bridging a portal area to centrilobular vein (technique: Sirius Red staining). **B)** Perisinusoidal/ pericellular fibrosis in a murine model of progressive NAFLD (technique: Sirius Red staining). **C.** Experimental biliary fibrosis (BDL or bile duct ligation) with ECM deposition in expanded periportal areas and ductular reaction (technique: immunohistochemistry for cytokeratin 19).

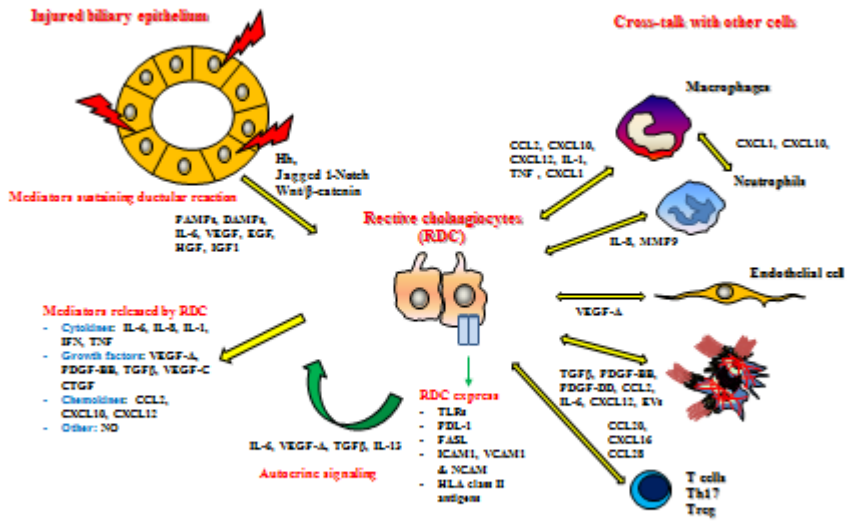


**Figure 4. Ductular reaction and reactive cholangiocytes: cellular interactions and mediators involved .** Following chronic injury to the biliary epithelium, ductular reaction occurs that involves so-called reactive cholangiocytes or reactive ductular cells (RDC). These cells in the scenario of chronic cholangiopaties release a number of mediators and express critical receptors or membrane proteins that allows an intense cross-talk with other cells in portal areas, including cholangiocytes themselves through autocrine signaling, macrophages, neutrophils, endothelial cells, portal fibroblasts or HSC sustaining their transdifferentiation into MFs, as well as different subsets of T lymphocytes (including Thelper 17 or Th17, and T regulatory or Treg cells. The release of extracellular vesiclesis is not here reported. Abbreviations: CCL, C–C motif chemokine ligand; CTGF, connective tissue growth factor; CXCL, C–X–C motif chemokine ligand; EGF, epidermal growth factor; ET1, endothelin 1; EVs, extracellular vesicles; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; Hh, hedgehog; ICAM1, intercellular cell adhesion molecule 1; IFN, interferon; IGF1, insulin-like growth factor 1; MMP, matrix metalloproteinase; NCAM, neural cell adhesion molecule; NO, nitric oxide; PDGF, platelet-derived growth factor; PD-L1, programmed cell death 1 ligand 1; TGF, transforming growth factor; TLRs, Toll like receptors; VCAM1, Vascular cell adeshion molecule 1; VEGF, vascular endothelial growth factor.

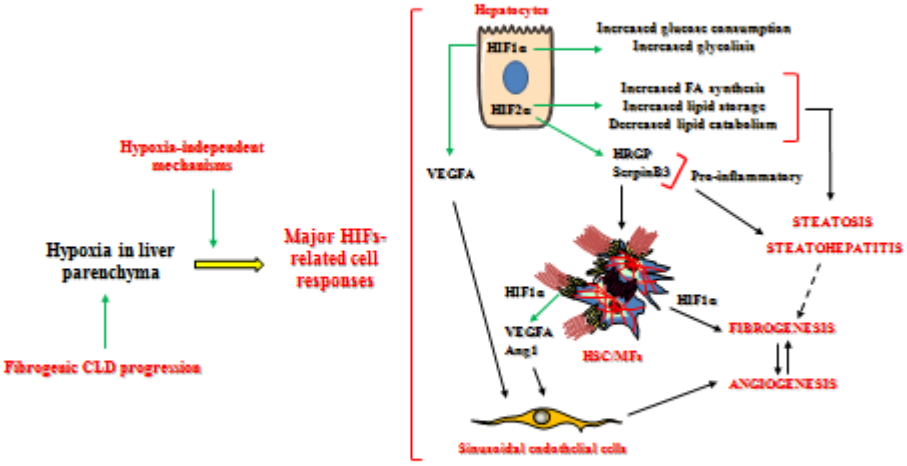


**Figure 5. Hypoxia- and HIFs-related roles in liver fibrogenesis.** Schematic and simplified analysis of major cellular responses that are believed to dependent on transcriptional activity of HIFs, either as a consequence of tissue hypoxia (very common in CLD progression) or of a number of hypoxia-independent mechanisms. The analysis of major HIFs-related cellular responses are intentionally limited to hepatocytes, sinusoidal endothelial cells and HSC activated to MF-like phenotype (HSC/MFs). More details in the text. Abbreviations: Ang1, angiopoietin 1; HRGP, histidine-rich glycoprotein; VEGFA, vascular endothelial growth factor A.