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REVIEW

Hepatic Stellate Cells: Dictating Outcome in Nonalcoholic Fatty Liver Disease

Leke Wiering,[1,2] Pallavi Subramanian,[3] and Linda Hammerich[1]

[1]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Department of Hepatology and Gastroenterology, Campus Virchow-Klinikum and Campus Charité Mitte, Berlin, Germany; [2]Berlin Institute of Health at Charité – Universitätsmedizin Berlin, BIH Biomedical Innovation Academy, BIH Charité Junior Clinician Scientist Program, Berlin, Germany; and [3]Institute for Clinical Chemistry and Laboratory Medicine, University Hospital and Faculty of Medicine, Technische Universität Dresden, Dresden, Germany

SUMMARY

Nonalcoholic fatty liver disease (NAFLD) frequently progresses to liver fibrosis, an important indicator of clinical outcomes. As hepatic stellate cell activation dictates fibrosis development during NAFLD, pathways that mediate hepatic stellate cell activation and inactivation ultimately determine course of disease in NAFLD.

Nonalcoholic fatty liver disease (NAFLD) is a fast growing, chronic liver disease affecting ∼25% of the global population. Nonalcoholic fatty liver disease severity ranges from the less severe simple hepatic steatosis to the more advanced nonalcoholic steatohepatitis (NASH). The presence of NASH predisposes individuals to liver fibrosis, which can further progress to cirrhosis and hepatocellular carcinoma. This makes hepatic fibrosis an important indicator of clinical outcomes in patients with NASH. Hepatic stellate cell activation dictates fibrosis development during NASH. Here, we discuss recent advances in the analysis of the profibrogenic pathways and mediators of hepatic stellate cell activation and inactivation, which ultimately determine the course of disease in nonalcoholic fatty liver disease/NASH. *(Cell Mol Gastroenterol Hepatol 2023;15:1277–1292; https://doi.org/10.1016/j.jcmgh.2023.02.010)*

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Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease worldwide with global prevalence rates of around 25% to nearly 30% in the adult population, which continue to increase.[1,2] Because targeted therapies are still lacking, NAFLD represents a high burden for those affected and for health care systems around the world.[3,4] NAFLD is considered the hepatic manifestation of the metabolic syndrome and is closely associated with diabetes mellitus, obesity, and dyslipidemia.[5] NAFLD encompasses several liver pathologies ranging from simple hepatic lipid accumulation (hepatic steatosis) to the more severe nonalcoholic steatohepatitis (NASH).[6] NASH is characterized by cellular stress, hepatocyte death, and inflammation, and may further progress to fibrosis, cirrhosis, and hepatocellular carcinoma.[7] The transition to fibrosis and cirrhosis is crucial because fibrosis is the key determinant of adverse outcomes and mortality and rates of liver-related complications increase with higher fibrosis stages.[3,8] The impact of NASH as a health burden is demonstrated by the fact that NASH is the fastest growing cause of hepatocellular carcinoma in patients listed for liver transplantation in the United States.[9] A key mechanism driving fibrosis development during NASH is the activation of hepatic stellate cells; once activated they differentiate into highly proliferative, extracellular matrix–producing myofibroblasts.[10] Therefore, understanding the underlying mechanisms and pathways of hepatic stellate cell activation is fundamental for developing effective therapies for NAFLD/NASH. This review provides an overview of the pathologic functions of hepatic stellate cells in the context of NAFLD and discusses the mechanisms involved in their activation and deactivation, and therapeutic approaches targeting these mechanisms.

Role of Hepatic Stellate Cells in Nonalcoholic Fatty Liver Disease

Etiology of Nonalcoholic Fatty Liver Disease

NAFLD is an umbrella term encompassing various liver pathologies including simple hepatic steatosis or nonalcoholic fatty liver and the more advanced NASH.[11] The pathogenesis of NASH is multifactorial, resulting from numerous conditions acting in parallel, such as abnormal lipid metabolism, genetic predisposition, lipotoxicity, oxidative stress,

Abbreviations used in this paper: **BAMBI, BMP and activin membrane-bound inhibitor; CCL2, chemokine (C-C motif) ligand 2; CCL3, chemokine (C-C motif) ligand 3; CCL5, chemokine (C-C motif) ligand 5; CCl_4, carbon tetrachloride; CTGF, connective tissue growth fact; EGF, epidermal growth factor; EP4, prostaglandin E2 receptor 4; GATA6, GATA-binding factor 6; GATA4, GATA-binding factor 4; IL, interleukin; MBOAT7, membrane-bound O-acyltransferase domain containing 7; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PGE2, prostaglandin E2; PPAR, peroxisome proliferator activated receptor; TCF21, transcription factor 21; TGF, transforming growth factor; TNF, tumor necrosis factor.**

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gut dysbiosis, endoplasmic reticulum stress, mitochondrial dysfunction, and inflammation.[11,12] NASH is associated with obesity and metabolic dysregulation, during which the adipose tissue exhibits low-grade inflammation and secretes adipokines and inflammatory cytokines, such as leptin, tumor necrosis factor (TNF), and interleukin (IL)6.[13] Furthermore, hepatic lipid accumulation correlates with insulin resistance, endoplasmic reticulum stress, mitochondrial dysfunction, and reactive oxygen species generation.[13,14] Additionally, NASH and NAFLD are associated with microbiota dysbiosis and a dysfunctional gut barrier resulting in an increased accumulation of gut-derived bacterial products, such as lipopolysaccharides and inflammation in the liver.[13,15]

During NAFLD, various intrahepatic and extrahepatic triggers lead to activation of liver-resident immune cells and to recruitment of additional immune cells of the innate and adaptive immune systems.[16,17] In particular, the liver resident macrophages named Kupffer cells play a major role in promoting inflammation during NAFLD.[18] The activation of Kupffer cells leads to the secretion of proinflammatory chemokines and cytokines, such as chemokine (C-C motif) ligand 2 (CCL2), CCL3, CCL5, TNF-α, IL1β, and IL6, which further aggravates inflammation by recruiting immune cells, such as monocyte-derived macrophages, neutrophils, and lymphocytes.[19-21] Although inflammation is an important mechanism of pathogenesis, patients tend to be mostly asymptomatic with respect to their liver disease at the earlier stages of NAFLD because the incidence for clinical symptoms increases with fibrosis stages.[3] This is in line with the findings that histopathologic inflammation alone is not a reliable predictor of NAFLD progression.[22] Fibrosis severity is the critical indicator of mortality during NASH, with higher risk of liver-related complications and death in NASH patients with F3 or F4 stage fibrosis.[3,23]

General Description of Hepatic Stellate Cells

Hepatic stellate cells are a nonparenchymal cell population, which represent the main fibrogenic cell type in the liver and account for approximately 5%–8% of all liver cells in the normal liver.[24,25] Hepatic stellate cells are localized in the space of Disse, between the basolateral region of hepatocytes and the antiluminal surface of the sinusoidal endothelial cells.[26] In the healthy liver, the resting, quiescent hepatic stellate cells have several cytoplasmic processes that aid in making contact with the surrounding cells, such as endothelial cells and hepatocytes, and assist in the intercellular transport of cytokines and soluble mediators.[26] A distinct feature of quiescent hepatic stellate cells is the storage of retinoids (vitamin A and its metabolites) within their cytoplasmic lipid droplets.[26,27] Under healthy conditions, approximately 80%–90% of the liver retinoids are stored in the lipid droplets of hepatic stellate cells.[26,27]

There is extensive crosstalk between hepatic stellate cells and immune cells, such as macrophages.[28,29] In vitro experiments using human macrophages and hepatic stellate cells show that soluble molecules derived from activated hepatic stellate cells induce the differentiation of macrophages to a proinflammatory phenotype.[28] Furthermore, recent single-cell RNA sequencing data suggest that hepatic stellate cells communicate with their surrounding endothelial cells and immune cells by secreting a variety of soluble factors or "stellakines," many of which are upregulated during NASH and liver injury. Thus, enhanced secretion of stellakines by hepatic stellate cells may be linked to NASH pathogenesis.[29]

Activation of Hepatic Stellate Cells

Hepatic stellate cells can be activated by various stimuli during liver injury and NASH (Figure 1).[30] Hepatic stellate cells produce most myofibroblasts during liver injury, as shown in rodent models of liver fibrosis and NASH.[25,31] The activation of quiescent hepatic stellate cells leads to their transdifferentiation into highly proliferative, extracellular matrix–producing activated hepatic stellate cells or myofibroblasts, with marked changes in their gene expression profile.[10,32] These activated hepatic stellate cells exhibit a contractile, proliferative, and fibrogenic phenotype, which can be further distinguished from quiescent hepatic stellate cells by the loss of their retinol-containing lipid droplets.[33] Although such activation represents a useful mechanism in acute injury, sustained activation of hepatic stellate cells as seen in NASH results in an excess accumulation of extracellular matrix that ultimately leads to fibrosis.[10] Activated hepatic stellate cells secrete a wide range of proinflammatory cytokines, such as CCL2, CCL5, IL8, chemokine (C-X-C motif) ligand-12 (CXCL12), and express adhesion molecules, such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 and chemokine receptors (CCR5), resulting in increased recruitment and infiltration of immune cells into the liver.[34]

Although quiescent hepatic stellate cells are rather homogeneous, activated hepatic stellate cells/myofibroblasts are much more heterogeneous.[29,35] Single-cell RNA sequencing studies performed in mouse and human livers have identified distinct subsets of resting and activated hepatic stellate cells/myofibroblasts in fibrotic and cirrhotic livers.[35-39] Functionally, quiescent hepatic stellate cells express high levels of growth factors and can protect hepatocytes from injury, whereas activated hepatic stellate cells mainly produce extracellular matrix proteins, such as different types of collagen I, but also collagen III, VI, and XIV, and cytokines and chemokines, promoting inflammation and fibrogenesis.[29,39] Furthermore, activated hepatic stellate cells can also exhibit immunoregulatory effects: hepatic stellate cells from human livers can induce apoptosis of activated T cells via programmed cell death 1 ligand 1; influence B-cell activity via the same mechanism in mice; and exert a positive influence on tolerancing immune, cells such as FoxP3$^+$ regulatory T cells or myeloid-derived suppressor cells in different mouse models.[40-43] Currently, it is not yet clear under which conditions hepatic stellate cells are proinflammatory and under which conditions they are tolerogenic. Several mechanisms trigger the activation of hepatic stellate cells during NAFLD pathogenesis, including metabolic injury with hepatocyte death and inflammation

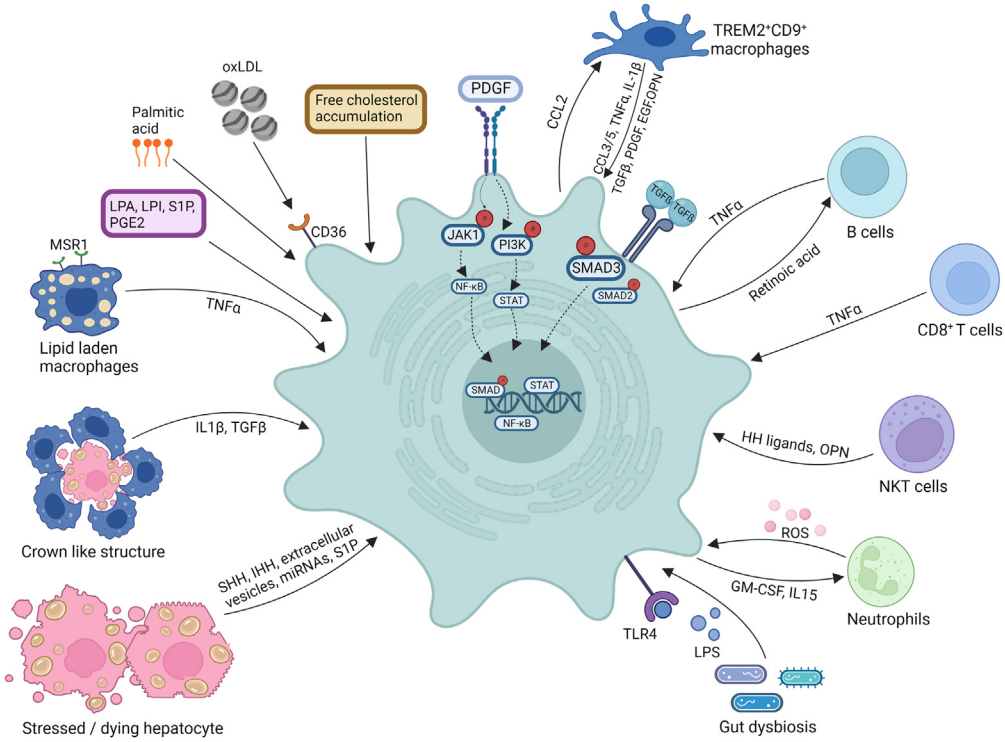

Figure 1. Pathways of hepatic stellate cell activation and survival during NAFLD. Various factors, such as lipid mediators, free cholesterol accumulation, oxLDL, palmitic acid, LPS, and immune cell–derived profibrotic molecules and growth factors, promote hepatic stellate cell activation and survival during NAFLD. OPN, osteopontin; HH ligands, hedgehog ligands; ROS, reactive oxygen species; GM-CSF, granulocyte macrophage colony-stimulating factor; LPS, lipopolysaccharide; TLR4, toll like receptor-4; SHH, sonic hedgehog; IHH, Indian hedgehog; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; S1P, sphingosine-1-phosphate; oxLDL, oxidized low-density lipoprotein; LPA, lysophosphatidic acid; LPI, lysophosphatidylinositol; MSR1, macrophage scavenger receptor 1; miRNA, microRNA. Created with Biorender.com.

with activation and recruitment of diverse immune cells.[33] The various triggers of hepatic stellate cell activation are described in detail in the later sections.

The activation of hepatic stellate cells is mainly mediated by growth factors, including transforming growth factor (TGF)β, platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) (Figure 1). TGFβ is the most potent activator of hepatic stellate cells and a key driver of fibrogenesis.[10] Of note, TGFβ levels are elevated in the serum of patients with NAFLD.[44,45] In the context of NAFLD, TGFβ originates mainly from immune cells, such as macrophages, and from activated hepatic stellate cells in a feed-forward loop.[46] During NAFLD, hepatocyte death and the resulting recruitment of macrophages is a critical driver of hepatic stellate cell activation and fibrosis via producing TGFβ.[10] When hepatocyte death occurs, recruited macrophages clear the apoptotic cells via the process of efferocytosis, which induces the production of immune regulatory factors, such as TGFβ by macrophages, leading to the activation of hepatic stellate cells.[10,47] Accordingly, inhibition of the TGFβ pathway in a mouse model of NAFLD results in reduced activation of hepatic stellate cells and attenuates fibrosis, an effect that was most pronounced when IL13 was inhibited simultaneously.[48] Mechanistically, TGFβ-induced hepatic stellate cell activation is mediated by the phosphorylation of SMAD proteins, such as SMAD 3, which ultimately leads to the upregulation of collagen I and III synthesis by complexes of phosphorylated SMAD molecules acting as transcription factors.[49-51] Genes that are upregulated by SMAD also include α-smooth muscle actin (α-*SMA*) and connective tissue growth factor (*CTGF*).[46] Furthermore, TGFβ can also induce activation of hepatic stellate cells in a SMAD-independent manner via mitogen-activated protein kinase-1, p38, and c-Jun N-terminal kinase mediated mechanisms, among others.[52,53] In addition to these direct effects, latent TGFβ can be deposited in the extracellular matrix and become subsequently activated by contraction of hepatic stellate cells through integrin-αV mediated mechanisms.[54,55] Concordantly, inhibition of integrin-αV has antifibrotic effects during liver injury.[54,55] Furthermore, the epigenetic regulator TET3 is involved in the TGFβ/SMAD2/3 activation pathway, which can be inhibited by microRNA-488-5p in mice with liver fibrosis induced by CCl_4 treatment, high-fat diet, or bile duct ligation.[56] Moreover, CTGF, also known as *CCN2*, is 1 of the target genes of TGFβ, which in addition to an increased secretion of extracellular matrix also promotes the proliferation and migration of hepatic stellate cells.[57] The expression of CTGF is relatively low in the healthy liver, but markedly increased in biopsies from patients with NAFLD.[58] Furthermore, silencing CTGF attenuates fibrosis development in a mouse model of CCl_4-induced liver injury.[59] In

addition to TGFβ-induced upregulation of CTGF, an additional TGFβ-independent upregulation via IL-13 has been described.[60,61]

PDGF is another profibrogenic growth factor. Under physiological conditions, PDGF is mainly produced by platelets; however, during liver injury PDGF is produced by macrophages, injured endothelial cells, and activated hepatic stellate cells.[33,62] During liver injury, there is also an increased expression of PDGF receptor-β (PDGFRβ) in hepatic stellate cells, which can promote increased signaling and activation of hepatic stellate cells.[63] Accordingly, in animal models of liver fibrosis, depletion of PDGFRβ leads to a reduction in fibrosis, conversely autoactivation of the receptor increases fibrosis development.[64,65] Moreover, PDGFRα is increased in biopsies of patients with severe NAFLD, and levels of circulating PDGFRβ may help predict liver fibrosis.[66,67] PDGF signaling is mediated by dimerization and subsequent autophosphorylation of the 2 PDGFRs after binding of a PDGF dimer.[68] PDGFRs are receptor tyrosine kinases and on activation trigger subsequent upregulation of mediators, such as phosphoinositide 3-kinase and Janus kinase-1.[62] Activation of PDGFR in hepatic stellate cells promotes their proliferation and migration; promotes cell survival; and increases the production of hedgehog pathway ligands, such as sonic hedgehog, which have been proposed to induce hepatic stellate cell activation.[69-71] Moreover, in patients with NASH, hedgehog activity correlates with the inflammation and fibrosis stage, whereas inhibition of hedgehog signaling reduces fibrosis in a mouse model of NASH.[72,73] Moreover, vascular endothelial growth factor has been shown to induce hepatic stellate cell activation and proliferation, thereby leading to enhanced fibrosis development.[74,75] The EGF receptor also seems to be involved in profibrotic pathways and is more strongly expressed in activated hepatic stellate cells.[76] Inhibition of EGF signaling results in decreased activation of hepatic stellate cells.[76,77]

Triggers of Hepatic Stellate Cell Activation in Nonalcoholic Fatty Liver Disease

Lipotoxicity. Hepatic lipotoxicity, caused by dysregulated lipid metabolism and enhanced influx of free fatty acids from peripheral tissues, is associated with NAFLD severity and progression.[78] Several toxic lipids, such as fatty acids, cholesterol, oxidized low-density lipoproteins, and others, promote macrophage activation, upregulate their proinflammatory phenotype, induce hepatic stellate cell activation (either directly or indirectly via increased inflammation, Figure 1), and promote NASH pathogenesis.[79-82] Free cholesterol is a key lipotoxic molecule in the context of NASH.[81,83] In animal models of NAFLD, several studies have demonstrated the importance of dietary cholesterol in promoting inflammation, fibrosis, and NASH pathogenesis.[83-85] Interestingly, dietary cholesterol and dietary fat act synergistically to aggravate the metabolic features of NASH in mice and trigger a hepatic pathology resembling that of advanced NASH in humans, which is characterized by increased macrophage recruitment, cholesterol dysregulation, production of oxidized low-density lipoproteins, and significant fibrosis.[84,85] Furthermore, free cholesterol is significantly increased in human NASH livers, whereas only a mild nonsignificant increase is seen in NAFLD samples.[86,87] Remarkably, cholesterol crystals accumulate in lipid droplets of steatotic hepatocytes, in mouse models of high fat, high cholesterol diet–induced NASH, and in human NASH, an event that is absent in samples with simple steatosis.[81,88] Steatotic, dead, or dying hepatocytes containing cholesterol crystals are encircled by Kupffer cells/monocyte-derived macrophages in an attempt to process the lipids, thereby resulting in the formation of activated, lipid-laden foamy macrophages and in the formation of the characteristic "crown like structures."[81,88,89] In this context, the Kupffer cells/monocyte-derived macrophages within the "crown like structures" get activated by cholesterol crystals leading to NLR family pyrin domain containing 3 (NLRP3) inflammasome activation and the production of proinflammatory factors, such as CCL2, IL1β, and TGFβ, which, along with amplifying inflammation, also promote hepatic stellate cell activation.[81,88,89] Furthermore, accumulation of free cholesterol in hepatic stellate cells leads to enhanced cell activation because of sensitization of hepatic stellate cells to TGFβ.[90] Free cholesterol accumulation in hepatic stellate cells promotes toll-like receptor-4 (TLR4) levels and signal transduction, which results in the suppression of hepatic stellate cell–specific BAMBI (a pseudoreceptor for TGFβ) and consequently TGFβ-induced hepatic stellate cell activation is boosted.[90]

In addition to directly activating hepatic stellate cells, toxic lipids can also be indirect activators of hepatic stellate cells, for instance via increasing inflammation. Lipid accumulation in macrophages enhances their inflammatory function.[80,82,91,92] Specifically, accumulation of lipid droplets in macrophages is important for the production of proinflammatory molecules, such as prostaglandin E_2 (PGE2), IL1β, and IL6.[91] Fatty acids, such as palmitic acid, can activate the TLR4-MD2 complex in macrophages to generate reactive oxygen species and pro-IL1β.[93] Additionally, palmitic acid impairs autophagy in macrophages via activation of hypoxia inducible factor-1α; the decreased autophagy together with upregulation of the hypoxia inducible factor-1α pathway leads to enhanced inflammation through the activation of nuclear factor-κB and increased production of CCL2 and IL1β.[94] Uptake of oxidized low-density lipoproteins by macrophages leads to lysosomal accumulation of these lipids and to increased inflammation.[82] In addition to macrophages, oxidized low-density lipoproteins also promote the activation of hepatic stellate cells, in cultured rat and human cells.[95,96] Oxidized low-density lipoproteins-induced hepatic stellate cell activation is mediated by the scavenger receptor CD36 (Figure 1).[96] Furthermore, in mouse macrophages, the scavenger receptor "macrophage scavenger receptor 1" (MSR1) facilitates palmitic acid–induced lipid accumulation and enhances *Tnfa* and *Il6* mRNA expression.[92] The macrophage-derived cytokines, such as TNFα and IL1β, in turn promote survival of activated hepatic stellate cells or myofibroblasts, as shown by in vitro and in vivo experiments.[97] Moreover, in a mouse

model of NAFLD, the PGE2/PGE2 receptor 4 (EP4) axis promotes hepatic stellate cell activation and fibrosis via activating the extracellular signal-regulated kinase pathway and enhancing autophagy in hepatic stellate cells.[98]

Although hepatocytes can accumulate cholesterol without necessary ballooning, this specific form of hepatocyte cell degeneration is an important histologic hallmark of NASH and is associated with fibrosis development.[99] Ballooned hepatocytes are profibrogenic and promote hepatic stellate cell activation possibly by producing sonic hedgehog.[100,101] Furthermore, hedgehog signaling has been suggested to promote glycolysis in hepatic stellate cells thereby regulating the myofibroblast phenotype.[102] Moreover, a direct link between NASH and hepatic cholesterol levels is well established.[81,103] Cholesterol accumulation in hepatocytes leads to hepatic stellate cell activation and NASH by stabilizing the transcriptional regulator TAZ (also known as WWTR1) in hepatocytes, resulting in the secretion of the profibrotic factor Indian hedgehog.[104] Sonic hedgehog, Indian hedgehog, and TAZ are upregulated in human livers affected by NASH but not simple steatosis,[105,106] suggesting a role of hepatocyte TAZ in promoting the transition from simple steatosis to NASH. Furthermore, fatty acids, such as palmitic acid, promote the release of extracellular vesicles from hepatocytes, which can be taken up by hepatic stellate cells resulting in their activation, both in human and mouse cells.[107] Hepatic stellate cells that internalize extracellular vesicles exhibit increased proliferation, chemotaxis, and enhanced expression of profibrotic genes, such as collagen, α-SMA, and tissue inhibitor of metalloproteinases-2.[107] Mechanistically, the extracellular vesicles contain microRNAs, such as miR-128-3p, and once transferred to the hepatic stellate cells, these microRNAs inhibit peroxisome proliferator activated receptor (PPAR)γ expression, ultimately leading to a switch from quiescent to activated hepatic stellate cell states.[107] Moreover, palmitate treatment of hepatocytes also results in the enhanced accumulation and secretion of the profibrotic lipid mediator, sphingosine-1-phosphate.[108] Of note, in addition to acting on hepatocytes, palmitic acid can also directly induce hepatic stellate cell activation via the inflammasome and possibly hedgehog signaling pathways.[109] Furthermore, steatotic hepatocytes upregulate Notch signaling, which correlates with NASH severity and results in the production of soluble mediators, such as osteopontin, which in turn activate hepatic stellate cells.[110,111] Consistently, inhibition of Notch signaling in hepatocytes reduces liver fibrosis. Additionally, dying hepatocytes also release damage-associated molecular patterns, such as P2Y14 ligands and the alarmin IL33, which activate mouse and human hepatic stellate cells through direct and indirect mechanisms and thereby promote fibrogenesis.[112,113]

Lipid Mediators. Profibrotic lipid mediators, such as lysophosphatidylinositol and lysophosphatidic acid, have been implicated in hepatic stellate cell activation and fibrogenesis (Figure 1).[114,115] Inhibition of the major lysophosphatidic acid generating enzyme autotaxin (ectonucleotide pyrophosphatase/phosphodiesterase family member 2) using a selective inhibitor leads to significant improvement in fibrosis development in mouse models of liver injury and NASH.[116] In addition, autotaxin levels are elevated in the serum of patients with NAFLD and patients with liver cirrhosis as compared with healthy individuals and correlate with the stage of fibrosis.[117,118] Consistently, in a model of CCl_4-induced liver injury in rats, circulating levels of lysophosphatidic acid and autotaxin activity are increased and correlate with the extent of liver fibrosis as well.[119] Moreover, circulating levels of lysophosphatidylinositol are increased in patients who have advanced fibrosis as compared with healthy individuals and the lysophosphatidylinositol-G protein-coupled receptor 55 axis has been shown to play a role in hepatic stellate cell activation.[115,120] Interestingly, the occurrence of the *MBOAT7 rs641738C>T* risk variant (which reduces membrane-bound O-acyltransferase domain containing 7 [MBOAT7] mRNA and protein) is associated with increased hepatic lysophosphatidylinositol levels and accordingly with enhanced NASH fibrosis.[121,122] Importantly, even in the absence of the *MBOAT7 rs641738C>T* risk variant, obesity suppresses hepatic MBOAT7 levels.[120] Therefore, the increased production and secretion of lysophosphatidylinositol because of reduced MBOAT7 levels may promote hepatic stellate cell activation, although further studies are required to demonstrate the underlying mechanisms.

The sphingosine-1-phosphate axis is also involved in hepatic stellate cell activation and hepatic fibrosis development.[108,123-125] In cultured human hepatic stellate cells, sphingosine-1-phosphate treatment directly induces cell activation, as shown by the upregulation of α-SMA expression.[108] Furthermore, sphingosine-1-phosphate treatment also induces the migration of human hepatic myofibroblasts in culture.[124] Moreover, in rodent models of bile duct ligation- or CCl_4-induced liver injury, treatment with the sphingosine-1-phosphate receptor 1/3 antagonist or with an inhibitor of sphingosine kinase (an enzyme that generates sphingosine-1-phosphate) suppress liver injury and hepatic fibrosis.[123] In addition, in a mouse model of diet-induced NASH, treatment with an sphingosine-1-phosphate antagonist abrogates NASH development[125] with reduced ballooning, fibrosis, and inflammation following feeding with a diet high in fat, fructose, and cholesterol.[125] Moreover, palmitate treatment of hepatocytes results in the increased accumulation of intracellular and extracellular sphingosine-1-phosphate levels.[108] Interestingly, conditioned medium derived from palmitate-treated hepatocytes promotes activation of hepatic stellate cells in culture and this activation can be blocked by cotreatment with an sphingosine-1-phosphate receptor 1/3 antagonist; suggesting that palmitate-treated hepatocytes release extracellular sphingosine-1-phosphate that can function in a paracrine manner to activate hepatic stellate cells.[108] In addition to promoting hepatic stellate cell activation, sphingosine-1-phosphate can also directly act on immune cells, such as macrophages, to promote NASH pathogenesis.[126] In a rodent model of diet-induced NASH, genetic or pharmacologic inhibition of the sphingosine-1-phosphate receptor 4 significantly reduces inflammation and NASH fibrosis via

mechanisms involving NLR family pyrin domain containing 3 (NLRP3) inflammasome activation in macrophages.[126]

Autophagy. Autophagy is a stress response mechanism that involves the degradation of cellular components and organelles through a lysosome-dependent pathway to generate energy and nutrients,[30] and plays a critical role in hepatic stellate cell activation and NASH fibrogenesis.[30,33,98,127-129] Autophagy is required to sustain an activated phenotype in hepatic stellate cells.[127,128] In culture-induced activation of mouse hepatic stellate cells a significant induction of autophagic flux is seen.[128] Moreover, treatment with an autophagy inhibitor reduces proliferation and expression of cellular activation markers, as shown in mouse and human hepatic stellate cells.[128] In mice containing autophagy defective hepatic stellate cells, CCl_4-induced liver injury results in abrogated extracellular matrix accumulation and fibrosis development.[127] Furthermore, the suppressed activation of hepatic stellate cells seen during autophagy deficiency can be partially rescued by the addition of exogenous fatty acids, such as oleic acid, therefore suggesting that the free fatty acids generated during autophagy are required to fuel cellular activation.[127] Several profibrotic/proinflammatory molecules, such as TGFβ and lipopolysaccharides, are known to upregulate autophagy in hepatic stellate cells.[129,130] TGFβ induced autophagy plays a role in hepatic stellate cell activation, possibly via the c-Jun N-terminal kinase and extracellular signal-regulated kinase signaling pathways.[129] Moreover, lipopolysaccharides-induced upregulation of autophagy mediates the suppression of the TGFβ pseudoreceptor BAMBI, thereby sensitizing hepatic stellate cells to TGFβ-induced cell activation.[130] Furthermore, macrophage-derived PGE2 can promote hepatic stellate cell activation and fibrosis by inducing autophagy.[98] In a mouse model of diet-induced NAFLD, M2-polarized macrophages induce hepatic stellate cell autophagy by producing PGE2, which acts via its receptor EP4 on hepatic stellate cell, consequently enhancing hepatic stellate cell activation, extracellular matrix production, and fibrosis development.[98] Blocking the PGE2/EP4 axis using an antagonist inhibits hepatic stellate cell autophagy and improves liver fibrosis.[98]

Endoplasmic reticulum stress has been shown to be upstream of autophagy in the hepatic stellate cell activation cascade.[30,131,132] In cultured hepatic stellate cells, overexpression of X-box binding protein 1, one of the unfolded protein response pathways, results in the upregulation of collagen expression; however, this induction of collagen is inhibited by deletion of an important autophagy mediator, autophagy related 7.[131] These data suggest that the X-box binding protein 1-mediated pathway contributes to fibrogenic activation of hepatic stellate cells and is linked to autophagy. Moreover, inositol-requiring enzyme 1, another component of the unfolded protein response pathway, induces hepatic stellate cell activation and autophagy, mediated via the p38 mitogen-activated protein kinase pathway.[132] Some of the key features of hepatic stellate cell activation are summarized in Figure 1.

Inflammation. During NAFLD, in addition to the metabolic triggers described previously, inflammation is a major cause of hepatic stellate cell activation.[10,33] Macrophages play a key role in the pathogenesis of NAFLD and are 1 of the cell types that interact with hepatic stellate cells.[10] Proinflammatory/profibrotic macrophages send strong activation and survival signals to hepatic stellate cells via secreting cytokines, such as TGFβ, PDGF, TNF-α, osteopontin, and IL1β, among others (Figure 1).[33,38,133] The chemokines CCL3 and CCL5 are also involved in hepatic stellate cell activation and fibrosis development during liver injury.[20,21] In mouse models of liver fibrosis, induced by carbon tetrachloride (CCl_4) treatment or by feeding with a methionine and choline-deficient diet, blocking CCL3 or CCL5 results in decreased activation of hepatic stellate cells and reduced fibrosis development.[20,21] Furthermore, in mouse and human tissues, single-cell RNA sequencing studies have identified the presence of a subset of macrophages that are $TREM2^+CD9^+$ and named as NASH-associated macrophages, lipid-associated macrophages, or scar-associated macrophages.[29,38,134] These $TREM2^+CD9^+$ scar-associated macrophages are particularly abundant in injured and cirrhotic human livers and secrete high levels of cytokines and growth factors including, EGF and PDGF-BB, osteopontin, and IL1β thereby providing a profibrogenic niche for hepatic stellate cells.[38] Furthermore, macrophages promote the survival of myofibroblasts via IL1 and TNF-dependent activation of the nuclear factor kappa-light-chain-enhancer of activated B cells pathway.[97] Moreover, the interaction between macrophages and hepatic stellate cells is not 1-sided; hepatic stellate cells also influence the differentiation of macrophages in the liver.[135] Activated hepatic stellate cells aggravate inflammation by inducing the proinflammatory polarization of macrophages via the p38 pathway and further promote the recruitment of monocyte-derived macrophages through secretion of CCL2.[28,136]

In addition to macrophages, other immune cells also provide activation signals to hepatic stellate cells (Figure 1). Immunodeficient animals with severe combined immunodeficiency or B cell deficiency show lower activation of hepatic stellate cells during liver injury. Transfer of $CD8^+$ T cells can reverse this effect.[137,138] Furthermore, B cells can promote NAFLD pathogenesis and hepatic stellate cell activation by secreting TNF-α and IL6, as shown in a mouse model of diet-induced NAFLD.[139] Of note, hepatic stellate cell-derived retinoic acid has been shown to augment B-cell survival.[140] Moreover, $CD8^+$ T cells are increased in different mouse models of NASH and in patients and can also have an activating effect on hepatic stellate cells through the secretion of profibrogenic cytokines, such as TNFα.[141,142] However, certain subsets of $CD8^+$ T cells have been shown to have an opposite effect, where $CD69^+CD103^-CD8^+$ tissue resident memory T cells can induce apoptosis of activated hepatic stellate cells via the FasL-Fas pathway.[143] T helper type 17 cells induce hepatic stellate cell activation through the production of IL17 and IL22, which stimulate TGFβ production in the liver and enhance TGFβ signaling and production of extracellular matrix molecules by hepatic stellate cells.[144] Natural killer T cell–derived hedgehog ligands and osteopontin can activate hepatic stellate cells.[145] Genetic deletion of natural killer T

cells leads to a decrease in fibrogenic factors in methionine-choline-deficient diet fed mice.[145] Neutrophil granulocytes can also trigger activation of hepatic stellate cells via secretion of their typical granules (eg, via myeloperoxidase).[146] With respect to the neutrophil-hepatic stellate cell crosstalk a positive feedback loop has also been described, in which hepatic stellate cells once activated by neutrophil-derived reactive oxygen species in turn improve neutrophil survival by producing granulocyte-macrophage colony-stimulating factor and IL15, thereby amplifying inflammation and fibrosis.[147] Additionally, extrahepatic proinflammatory signals can act as triggers for hepatic stellate cell activation, because quiescent hepatic stellate cells express various receptors of the innate immune system including TLR4, which can be activated by lipopolysaccharides.[148] This is of particular relevance, because patients with NAFLD often show alterations of the gut-liver-axis with intestinal dysbiosis and increased translocation of intestinal bacteria.[149] Furthermore, it has been shown in mice that stimulation of quiescent hepatic stellate cells with lipopolysaccharides induces cell activation resulting in increased chemokine secretion, positively affecting chemotaxis of Kupffer cells/macrophages, and increasing the susceptibility to TGFβ signaling through downregulation of the BMP and activin membrane-bound inhibitor (BAMBI; TGFβ pseudoreceptor).[150] Although the most relevant aspect of TGFβ signaling in the context of this review may be activation of hepatic stellate cells and therefore its profibrogenic role as described previously, it is also worth mentioning the interactions TGFβ shows with various immune cells. TGFβ has been described to promote the differentiation of FoxP3$^+$CD4$^+$ regulatory T cells, which was also associated with the progression of hepatocellular carcinoma.[151] This immunosuppressive effect of TGFβ seems to be mediated via retinoid metabolism by hepatic stellate cells.[152] Because activated hepatic stellate cells lose their lipid droplets this may explain why this mechanism of liver-induced tolerance by TGFβ is not sufficiently protective against inflammation in the presence of continuous stimuli as in NASH. TGFβ can also promote a more anti-inflammatory/immunosuppressive phenotype in macrophages, sometimes called M2 or alternately activated macrophages.[153]

Inactivation of Hepatic Stellate Cells During Nonalcoholic Fatty Liver Disease

Reversal of hepatic stellate cell activation has been demonstrated in rodent models of fibrosis resolution.[154,155] During fibrosis regression activated hepatic stellate cells are removed either via apoptosis or by reverting to a quiescent-like phenotype (Figure 2), where they remain in a primed state and respond more rapidly to new fibrogenic stimuli, thereby contributing to faster fibrosis development on reinjury.[154-156] Several transcription factors, such as PPARγ, GATA-binding factor 6 (GATA6), GATA-binding factor 4 (GATA4), and transcription factor 21 (TCF21), have been implicated in regulating the deactivation of hepatic stellate cells.[157-159] In a model of CCl_4-induced liver injury, mice

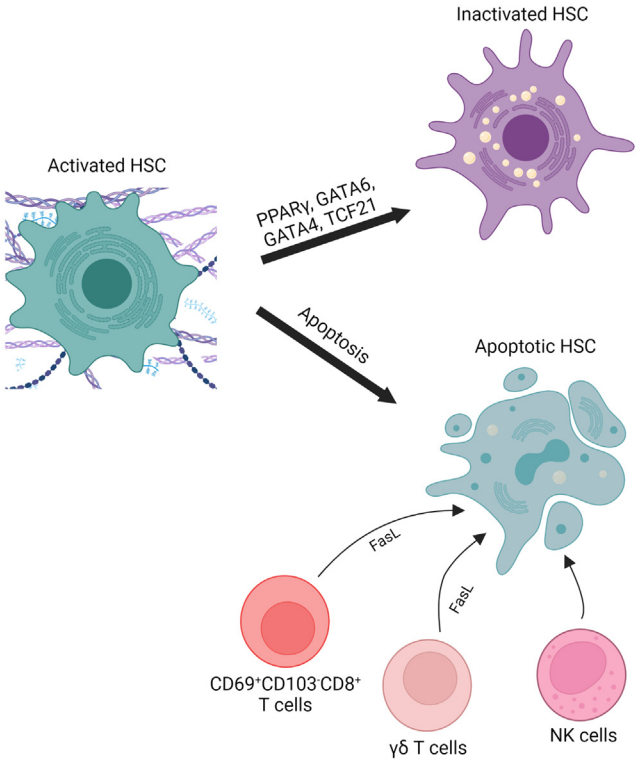

Figure 2. Inactivation of hepatic stellate cells during NAFLD. Activated hepatic stellate cells that accumulated during NASH fibrosis are removed during fibrosis resolution either by apoptosis or by reverting to an inactive phenotype. Additionally, activated hepatic stellate cells are removed by cell death induced by immune cells such as CD69+CD103-CD8+ T cells, $\gamma\delta$ T cells and NK cells. The transcription factors PPARγ, GATA6, GATA4 and TCF21 play an important role in promoting and maintaining the inactivated phenotype of hepatic stellate cells. FasL, Fas ligand; HSC, hepatic stellate cells; NK, natural killer; TCF21, transcription factor 21. Created with Biorender.com.

with hepatic stellate cell–specific deletion of GATA6 or PPARγ are more susceptible to fibrosis development.[159] Moreover, regression of liver fibrosis is significantly reduced in hepatic stellate cell–specific GATA6-deficient mice and in hepatic stellate cell–specific PPARγ-deficient mice as compared with wild-type mice, therefore suggesting that both GATA6 and PPARγ are critical for maintaining the inactivated phenotype of hepatic stellate cells.[159] Furthermore, the expression of the transcription factor TCF21 is diminished in activated hepatic stellate cells of methionine and choline-deficient diet or CCl_4-treated fibrotic mouse livers as compared with normal livers.[158] This reduced TCF21 expression in hepatic stellate cells is restored during regression of liver fibrosis.[158] Overexpression of TCF21 in activated hepatic stellate cells/myofibroblasts downregulates the mRNA expression of profibrotic genes, such as *Col1a1* (encoding for collagen, type I, alpha 1), *Pdgfrb* (encoding for PDGFR-beta), and *Acta2* (encoding for α-SMA); in contrast, the expression of hepatic stellate cell quiescence genes, such as *Gfap* (encoding for glial fibrillary acidic protein) and *Ngfr* (encoding for nerve growth factor

Table 1. Clinical Trials With Antifibrotic Agents in Liver Fibrosis

Compound	Type	Phase	Trial number	Patients	Status	Treatment duration	Main result
Maraviroc	CCR-5 inhibitor	IV	NCT03129113	NAFLD and HIV	Completed	48 wk	No reduction of hepatic fat by MR-PDFF[165]
Belapectin	Galectin 3 inhibitor	IIb	NCT02462967	NASH F4 cirrhosis with portal hypertension	Completed	52 wk	No reduction of HPVG, no histologic improvement[169]
Simtuzumab (GS-6624)	Monoclonal antibody against LOXL2	IIb	NCT01672866	NASH F3/4 fibrosis	Completed	96 wk	No histologic improvement of hepatic collagen[170]
Simtuzumab (GS-6624)	Monoclonal antibody against LOXL2	IIb	NCT01672879	NASH F4 cirrhosis	Completed	96 wk	No reduction of HPVG[170]
Pirfenidone	Antifibrotic agent	II	NCT02161952.	Chronic hepatitis C	Completed	24 mo	Significant histologic improvement[171]
Pirfenidone	Antifibrotic agent	II	NCT04099407	Advanced fibrosis (mixed etiologies)	Completed	12 mo	Significant reduction of fibrosis by noninvasive measurement[172]
BMS-986263	HSP47 siRNA	II	NCT03420768	Hepatitis C F3/4 fibrosis	Completed	12 wk	Association with histologic improvement[173]
BMS-986263	HSP47 siRNA	II	NCT04267393	NASH compensated F4 cirrhosis	Recruiting		
PLN-1474	Integrin $\alpha V\beta 1$ inhibitor	I	not available	NASH F3/4 fibrosis	Recruiting		

CCR-5, C-C chemokine receptor type 5; HPVG, hepatic venous pressure gradient; HSP47, Heat shock protein 47; LOXL2, Lysyl oxidase homolog 2; MR-PDFF, magnetic resonance proton density fat fraction; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

receptor), are upregulated.[158] Therefore, TCF21 acts as a deactivation factor in fibrogenic hepatic stellate cells. Furthermore, GATA4 is another transcription factor that is involved in the suppression of hepatic stellate cell activation.[157] Accordingly, overexpression of GATA4 in the liver promotes the regression of CCl_4-induced liver fibrosis.[157]

Immune cells are also involved in suppressing hepatic stellate cell activity by inducing cell death (Figure 2).[143,160] Specifically, $\gamma\delta$ T cells, certain $CD8^+$ T cells, and natural killer cells can have a proapoptotic effect on hepatic stellate cells and thus an antifibrotic effect.[143,160,161] Hepatic $\gamma\delta$ T cells induce apoptosis of hepatic stellate cells in a cell-to-cell contact-dependent mechanism and involving Fas-ligand, as shown in mouse models of liver injury.[160] Similarly, $CD69^+CD103^-CD8^+$ tissue resident memory T cells promote apoptosis of activated hepatic stellate cells in Fas-ligand dependent fashion.[143] Moreover, natural killer cells also mediate an antifibrotic effect through inducing apoptosis of hepatic stellate cells.[162] Activation of natural killer cells in mice with liver fibrosis attenuates fibrosis development by inducing cell death of activated hepatic stellate cells, whereas depletion of natural killer cells reverses this effect, a mechanism mediated by retinoic acid early inducible 1 and TNF.[161]

Targeting Hepatic Stellate Cells

Despite the increasing prevalence rates and intensive research, there is still no approved NASH-specific therapy available. The agents investigated in advanced clinical trials so far aim at improving metabolic injury (eg, PPAR agonists, farnesoid X receptor agonists, thyreomimetics, glucagon-like peptide agonists and others), reducing hepatocyte injury (eg, ASK-1 inhibitors and caspase inhibitors) and inhibiting immune cell activation and recruitment (eg, CCR2/5 antagonists, PPAR agonists, farnesoid X receptor agonists). As described previously, these factors are important triggers for hepatic stellate cell activation, and pharmacologic manipulation of the upstream mediators or cells may lead to reduced activation of hepatic stellate cells. Specific effects on hepatic stellate cells have also been described for some of these substances; relevant clinical trials are summarized in Table 1. As mentioned, CCL5 is involved in the activation of hepatic stellate cells[163] and CCR-5 inhibitors, such as maraviroc, might therefore have antifibrotic effects. In vitro experiments with human hepatic stellate cells showed a reduction of collagen and extracellular matrix and TGFβ as an important mediator of hepatic stellate cell activation.[164] However, clinical trials have only been conducted in a small cohort of patients with HIV with NAFLD, because maraviroc is already approved as a combination therapy in HIV treatment. In this study, no reduction in hepatic fat could be detected radiographically, but the applicability to other patient populations is uncertain.[165] Although PPAR agonists are thought to act primarily by influencing metabolism in hepatocytes, PPARβ, γ and δ are also expressed in hepatic stellate cells. Their effects in hepatic stellate cells are not yet comprehensively understood, but there is evidence that PPARγ, for example, has inhibitory effects on hepatic stellate cell activation and proliferation and can promote their inactivation.[159,166,167] Reduced synthesis of extracellular matrix has also been described for glucagon-like peptide agonists, at least in NASH animal models.[168]

In addition, substances with a more primary effect on hepatic stellate cells are also investigated. Galectin 3 is a cytosolic protein in cells of the innate immune system, especially in macrophages. Increased expression of galectin 3 causes activation of myofibroblasts and galectin 3 has profibrotic effects in various organs.[174] In animal studies, galectin 3 inhibitors have been shown to reduce fibrosis.[175] Belapectin is a pharmacologic inhibitor of galectin 3 that has been previously studied in clinical trials in patients with NASH. In a phase IIb study, no histologic improvement in fibrosis was achieved after 1 year.[169] However, subgroup analysis showed improvement in hepatic stellate cells in patients without preexisting varices, and further studies are now ongoing for this subgroup. Another antifibrotic agent currently in clinical trials is BMS-986263, a nanoparticle containing HSP47 siRNA. HSP47 for its part is a serine proteinase inhibitor, a protein that specifically binds collagen as a so-called chaperone.[176] Thus, by binding of the siRNA, collagen synthesis can be negatively affected. First results of the phase II study in patients with cured hepatitis C but advanced fibrosis (stage 3 or 4) were published in early 2022 and showed histologic improvement of fibrosis.[173] Currently, recruitment is underway for NCT04267393, a phase II study to test BMS-986263 in patients with NASH with compensated cirrhosis (F4). After synthesis, collagen is cross-linked by lysyl oxidases, resulting in greater stability. Therefore, another antifibrotic therapeutic approach is the inhibition of these enzymes. Simtuzumab is a monoclonal antibody against Lysyl Oxidase Like 2 (LOXL2), which prevents LOXL2-mediated stabilization of the extracellular matrix and was able to accelerate fibrosis resolution in mouse models.[177] However, in 2 phase IIb studies in patients with advanced NASH fibrosis and NASH cirrhosis, respectively, no significant effect was observed after 96 weeks of treatment.[170] It is possible that other lysyl oxidases may play a more relevant role.

Other fibrosis-specific therapeutic approaches are based on influencing TGFβ. This can be activated in its latent form in an integrin-mediated manner, as already mentioned. Inhibition of integrin-αV by a pan αV inhibitor causes attenuation of fibrosis in various organ models including liver.[178,179] Other integrin antibodies are already approved for different diseases (eg, vedolizumab for inflammatory bowel disease) and clinical trials are ongoing for idiopathic pulmonary fibrosis, but clinical trials in liver fibrosis are still lacking. Currently, only a phase I trial of the selective $\alpha V\beta 1$ inhibitor PLN-1474 in patients with NASH has been announced.[180] Further interference with TGFβ may be feasible with pirfenidone, an oral antifibrotic agent already approved for the treatment of idiopathic pulmonary fibrosis. The exact mechanism of action is not yet clear, but preclinical studies show reduced collagen synthesis and lower TGFβ levels.[181,182] A small clinical trial in patients with chronic hepatitis C showed histologic improvement in fibrosis and also reduced TGFβ levels after 2 years of

treatment.[171] In the more recent PROMETEO study in patients with advanced fibrosis, of which the largest group was NASH-related, a reduction in fibrosis was also seen, but noninvasive measurement was used.[172] Other growth mediators may also be modulated. Angiotensin receptor antagonists are an established therapy option for cardiovascular disease and there is some evidence from rat models that these agents attenuate activation of hepatic stellate cells.[183,184] Angiotensin II has also been shown to cause upregulation of CTGF and downstream activation of SMAD2/3,[185] but human studies on the effect of these agents in NAFLD are lacking. An ameliorating effect on the development of NAFLD has also been described for EGF receptor. In NAFLD mouse models, continuous administration of canertinib, an EGF receptor inhibitor, prevented the development of steatosis. Additionally, in animals with preexisting NASH, this therapy significantly reduced fibrosis.[186] Other studies show a reduced number of activated hepatic stellate cells after administration of an EGF receptor inhibitor in animal models of progressive cirrhosis.[76] However, human studies on these compounds are also lacking to date.

Recently, obeticholic acid has been resubmitted to the Food and Drug Administration for approval as the first specific drug to treat NASH fibrosis.[187] However, specific antifibrotic therapies are still lacking. It remains to be seen how the new insights into hepatic stellate cells from single cell analyses described previously can be translated into therapeutic concepts.

Conclusions and Future Perspectives

The prevalence of NAFLD is growing rapidly, with fibrosis severity as the critical determinant of disease progression and mortality. Hepatic stellate cells are the precursors for most profibrogenic, extracellular matrix producing myofibroblasts during NAFLD. Various factors, such as inflammation, lipotoxicity, lipid mediators, and growth factors, can promote hepatic stellate cell activation during NAFLD (Figure 1). Additionally, the recruitment and activation of profibrogenic macrophages in response to hepatocyte death is crucial for triggering hepatic stellate cell activation and survival as outlined in the accompanying review by Vonderlin and colleagues. In summary, recent studies have expanded the knowledge on the pathways and mechanisms of hepatic stellate cell activation and NASH fibrosis. However, further studies are required to establish the best intervention to inhibit and reverse the fibrogenic process.

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Correspondence
Address correspondence to: Linda Hammerich, PhD, Charité-Universitätsmedizin Berlin, Department of Hepatology and Gastroenterology, Campus Virchow-Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany. e-mail: Linda.hammerich@charite.de.

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CRediT Authorship Contributions
Leke Wiering, MD (Conceptualization: Equal; Data curation: Equal; Visualization: Lead; Writing – original draft: Equal; Writing – review & editing: Equal)
Pallavi Subramanian, PhD (Conceptualization: Equal; Visualization: Supporting; Writing – original draft: Equal; Writing – review & editing: Supporting)
Linda Hammerich, PhD (Conceptualization: Equal; Visualization: Supporting; Writing – original draft: Equal; Writing – review & editing: Lead)

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