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Mechanisms of Fibrosis Development in NASH

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent liver disease world-wide, affecting 20–25% of the adult population. In 25% of patients, NAFLD progresses to non-alcoholic steatohepatitis (NASH), which increases the risk for the development of cirrhosis, liver failure and hepatocellular carcinoma. In patients with NASH, liver fibrosis is the main determinant of mortality. Here, we review how interactions between different liver cells culminate in fibrosis development in NASH, focusing on triggers and consequences of hepatocyte-macrophage-hepatic stellate cell (HSC) crosstalk. We will discuss pathways through which stressed and dead hepatocytes instigate the profibrogenic crosstalk with HSC and macrophages including the reactivation of developmental pathways such as TAZ, Notch and hedgehog; how clearance of dead cells in NASH via efferocytosis may affect inflammation and fibrogenesis; and insights into HSC and macrophage heterogeneity revealed by single cell RNA-sequencing. Finally, we will summarize options to therapeutically interrupt this profibrogenic hepatocyte-macrophage-HSC network in NASH.

BACKGROUND

With nearly 40% of the world's population being overweight or obese, non-alcoholic fatty liver disease (NAFLD) is becoming a rapidly growing health problem, affecting ≈25% of the world's adult population[1], i.e. ~1.5 billion people. Chronic liver disease (CLD) due to non-alcoholic steatohepatitis (NASH), an advanced form of NAFLD, is expected to become the leading cause of liver transplantation in the US[2,3], whereas CLD caused by HBV and HCV are declining due to improved treatments and HBV vaccination programs. Despite the extent of the problem, there are currently no approved therapies for NAFLD and NASH[4,5]. With several studies showing fibrosis as main determinant of mortality in NASH[3,6,7], fibrosis has

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become a major focus in the NASH arena. Moreover, regulatory agencies such as FDA and EMA are mandating trials in advanced NASH so that the effect of interventions on measurable outcomes, including fibrosis, can be determined[8,9]. While correcting the underlying metabolic alterations is likely the best treatment for NAFLD, hepatocytes are in part replaced by fibrotic scar tissue in advanced NASH and are severely altered, rendering therapeutic targeting of underlying metabolic abnormalities less efficient. Therefore, increasing emphasis has been put on therapies that improve NASH fibrosis - which may be achieved by improving metabolic abnormalities, liver injury or inflammation, and/or by direct antifibrogenics[5]. Additional targets lie outside of the liver, such as the microbiome/gut-liver axis to decrease energy extraction or improve inflammation[5,10–12]; the CNS to limit food intake[5]; adipose and muscle to improve metabolism and inter-organ crosstalk[13,14]; and kidney to lose calories, e.g. via SGLT2 inhibition[15].

Fibrosis is the result of excessive production of extracellular matrix (ECM) that is not adequately balanced by degradation, thus resulting in net accumulation. In the liver, hepatic stellate cells (HSC) constitute the main source of ECM-producing fibroblasts in models of toxic and biliary liver disease and NAFLD[16–19]. Portal fibroblasts represent only a minor source of liver fibroblasts in most studies[18,19] and they are not known to play a role in NASH. ECM represents a complex network of ECM proteins that include 20 genetically distinct types fibrillary and non-fibrillar collagen; noncollagenous glycoproteins such as elastin, laminin, and fibronectin; glycosaminoglycans such as hyaluronan; proteoglycans such as aggrecan, fibromodulin, decorin, biglycan, glypicans, and syndecans [20]. In addition to increased amounts, the composition of the ECM also changes in fibrosis[20,21], with increases in embryonic or wound-healing associated ECM and increased crosslinks, which render ECM more resistant to degradation, contributing to the slow and often incomplete reversibility of advanced fibrosis. Besides acting as a three-dimensional scaffold that provides structure and determines boundaries, ECM provides important cues to surrounding cells. Notably, ECM controls cell survival, proliferation and differentiation and possibly metabolic pathways, acting through via specific cell surface receptors such as integrins or via the modulation of mechanosensitive signaling pathways[22,23]. Moreover, matricellular proteins such as thrombospondins, connective tissue growth factor, osteopontin and ECM-associated growth factors such as HGF may also affect hepatocytes and non-parenchymal cells. While alterations of the liver “matrisome” have been described in alcoholic liver disease [21], its composition in NASH remains to be determined. Hence, altered ECM composition in the fibrotic or cirrhotic NASH liver can impact liver function, regeneration and carcinogenesis[24,25]. The majority of clinical studies focus on complications associated with fibrosis, such as the replacement of liver parenchyma (contributing to liver failure) as well as increased stiffening and vascular resistance (contributing to portal hypertension). However, the underlying pathobiology is complex and not fully understood, and may also encompass protective functions of HSC and ECM/matrisome. It is conceivable that the role of HSC and ECM in NASH changes from restorative in early stages to disease-promoting in later stages. While fibrosis and cirrhosis are viewed as the common end-stage of different forms of CLD, it is not known whether there are disease-specific characteristics in regards to HSC activation, ECM and matrisome relevant for human NASH.

Hepatic stellate cell activation in NASH

HSC represent the dominant hepatic fibrogenic cell population contributing about 80–95% of collagen-producing myofibroblasts in different mouse models of fibrosis including NASH[18]. HSC are responsible for most of the architectural changes that characterize the fibrotic or cirrhotic NASH liver, in particular the deposition of the type I collagen-rich ECM, which contributes to typical complications such as portal hypertension and loss of functional liver mass. With HSC activation and fibrogenesis representing a unifying element in the response to hepatic injury between different liver diseases, it is conceivable that HSC activation is a conserved process, retaining high similarities between different liver diseases. This concept has been suggested for BDL- and CCl_4-induced liver injury[26] and recently for NASH-driven fibrosis[27]. At the same time, recent single cell RNA-sequencing (scRNA-seq) studies have revealed heterogeneity within the fibrogenic cell population, even though this needs further confirmation in larger cohorts. In patients, scRNA-seq has showed multiple profibrogenic cell populations including HSC, mesothelia/portal fibroblasts, vascular smooth muscle cells and scar-associated mesenchymal cells[28]. scRNA-seq in mice has shown fibrogenic cells to largely consist of HSC and only a small proportion of a portal fibroblast-like cell population in multiple models including NASH fibrosis[29] (and unpublished results, RFS). Moreover, there is cellular heterogeneity in regards to activation and proliferation in both mice and patients[28,29] as well as heterogeneity linked to anatomical localization in specific zones[30] or interaction with other cell types such as scar-associated macrophages in specific locations[28]. While some of this heterogeneity may reflect the transition between different states (resting vs proliferating vs activated), it is likely that there is also functional heterogeneity. Hence both a fibrogenic core program, operating in virtually all HSC, as well as disease-, location-, context- or patient-specific functions may exist in parallel.

Transforming growth factor-β (TGFβ) is the most potent fibrogenic cytokine and a key driver of HSC activation and liver fibrosis[31]. TGFβ is released in its latent form by several hepatic cell populations[31], and is locally activated by HSC expressing integrin aV[32]. Profibrogenic effects of TGFβ are mediated by SMAD-dependent pathways, by MAPK[17] and possibly by YAP-dependent pathways[33]. Similar to toxic and biliary liver fibrosis, pharmacologic inhibition of TGFβ reduces NASH-induced fibrosis, albeit only partially, and combined inhibition of TGFβ and IL-13 signaling attenuates the fibrotic machinery more efficiently than TGFβ alone[34]. Platelet-derived growth factor (PDGF) represents a second fibrosis-promoting cytokine[31]. HSC express high levels of PDGF receptors, whose activation potently stimulates HSC proliferation and migration[31]. However, the specific role of PDGF signaling in NASH remains to be established. Emerging data show a key role of YAP in HSC activation as demonstrated by reduced fibrosis in mice treated with YAP inhibitor verteporfin and reduced HSC culture activation treated with YAP siRNA or verteporfin[35,36]. As YAP is also strongly upregulated in hepatocytes and cholangiocytes, it is not clear to what degree the reduction of fibrosis in verteporfin-treated mice is due to inhibition of YAP in HSC versus other cell types. Moreover, the contribution of HSC YAP/TAZ to NASH fibrosis as well as triggers of YAP/TAZ activation in HSC remain elusive. Other activators of HSC in NASH include Indian hedgehog (IHH, induced by TAZ in hepatocytes), sonic hedgehog (SHH, expressed by ballooning hepatocytes) and osteopontin (induced by hepatocyte Notch and TAZ)[37–39]. A plethora of additional

mediators including CTGF, Il-17 and angiotensin II also contributes directly to HSC activation[17,40,41] but their role in NASH remains to be determined. In summary, it appears that cellular events in hepatocytes and possibly also macrophages differ substantially between NASH and other liver diseases, whereas the activation of HSC may follow a more conserved pattern with similarities between NASH and other liver diseases and more subtle disease- and/or patient-specific variations. However, further studies in mice and patients are needed to substantiate this concept, in particular larger-scale scRNA-seq studies that compare various cell populations in patients with different liver diseases.

Cellular networks driving HSC activation and fibrosis as well as fibrosis resolution in NASH.

Cell death and inflammation are key drivers of fibrosis in NASH and other forms of CLD[42–44]. Both are triggered, sensed and responded to by cellular networks consisting of distinct resident and non-resident cells (Fig.1). Accordingly, cellular networks - rather than a single cell type – regulate fibrosis development in NASH. While our review will focus on the hepatocyte-macrophage-HSC network as most important driver of fibrosis in NASH, several other cell populations affect HSC activation and fibrosis in NASH[42] (Fig.1). The contribution of immune cells is best exemplified by the decrease of CCl_4- and NASH-induced liver fibrosis in RAG2−/−[42] and RAG1−/− mice[45], which lack mature B, T and NKT cells. As such B cells[46], NKT cells[45,47,48], platelets[49,50] and type 2 innate lymphoid cells (ILC2)[51] promote liver fibrosis, whereas NK cells may restrict fibrosis via killing of HSC[52–54]. In NASH, NKT cell promote fibrosis by increasing steatosis and hepatocellular damage[45,48]. Platelets promote NASH fibrosis by increasing lipid accumulation and immune cell infiltration[49]. Moreover, LSEC normally suppress HSC activation and lose this suppressive state in fibrosis[55,56]. While LSEC acquire lose their fenestrae at early stages of NAFLD[57,58], their functional role in NASH fibrosis remains to be determined.

Similar to other liver diseases (i.e. viral hepatitis, biliary obstruction or autoimmune hepatitis), successful treatment of the underlying disease results in fibrosis regression, as demonstrated in patients undergoing bariatric surgery[59–62]. Unlike patients with viral hepatitis, where successful treatment of the underlying disease can lead to the reversal of cirrhosis, such data are currently not available for NASH. Similar to the multi-cellular network involved in fibrogenesis, fibrosis resolution involves multiple cell types, in particular pro-resolution and ECM-degrading macrophages, promoting HSC apoptosis[63] and HSC deactivation[64,65] as well as the degradation of ECM[66]. These underlying mechanisms and cell-cell interactions in fibrosis regression have been reviewed in detail elsewhere[66,67] and there is currently no insight whether fibrosis regression, HSC apoptosis and deactivation in NASH differs from other diseases. The fact that alterations of hepatocytes (via improving hepatocyte health), macrophages (via a switch from fibrotic to fibrosis-resolving macrophage) and HSC (via HSC apoptosis and deactivation) contribute to fibrosis resolution suggest that the hepatocyte-macrophage-HSC network not only stands in the center of fibrogenesis but represents the core network in fibrosis resolution. While there are additional signals feeding into this cellular network such as above-discussed liver cell types, the gut-liver axis - via microbial signals and metabolism, FXR activation and FGF19[68] - as well as the adipose tissue - via adipokines and possibly via newly identified adipocyte-released

lipid-filled vesicles (AdExos) that affect macrophage differentiation[69] - we will focus on cell-cell crosstalk within the liver as well as means of therapeutically targeting it in this review.

How stressed, "undead" and dead hepatocytes trigger HSC activation and fibrosis in NASH

Hepatocytes contribute to HSC activation via multiple mechanisms. Most notably, hepatocyte stress and death promote inflammation, resulting in recruitment of macrophages and their secretion of profibrogenic mediators such as TGFβ, thus putting the hepatocyte-macrophage-HSC network at the center of the fibrogenic response in NASH (Fig.1). However, there is also evidence for HSC activation occurring through direct interactions of stressed or dead hepatocytes with HSC (Fig.1). This may be through the release of profibrogenic DAMPs[43,44,70] or other profibrogenic mediators such as Hh ligands and osteopontin[37–39], or via apoptotic bodies[71,72], which may directly act on HSC. It is possible that (i) these are relevant in settings where there is little macrophage-derived TGFβ pathway, (ii) they occur in parallel to TGFβ-mediated HSC activation, resulting in stronger fibrogenesis, or (iii) hepatocyte-derived signals amplify TGFβ activation or TGFβ signaling in HSC.

Hepatocyte death as trigger for cell-cell networks that promote fibrosis.— Hepatocellular stress and death and the subsequent induction of fibrosis is common to all types of CLD including NASH. Similar to chronic HBV and HCV infection[73–78], elevated levels of serum ALT, a surrogate marker for hepatocyte death, predict the presence of liver fibrosis and risk for fibrosis progression in NASH[79,80]. Vice versa, normalization of ALT levels after lifestyle intervention is significantly associated with fibrosis improvement in NASH[81]. As one third of patients with NASH fibrosis have normal ALT, it appears that although hepatocyte death is associated with NASH and fibrosis development, current methods to detect cell death are not sufficiently sensitive and/or that additional events may drive disease progression. Hepatocyte death comes in many flavors including apoptosis, necroptosis, necrosis, pyroptosis and ferroptosis. While it was initially suggested that apoptosis is the most prominent form of cell death in NASH[82] and knockout of caspase 3 protected from several - but not all - aspects of MCD-induced NASH[83], recent studies have also found evidence for necroptosis in murine and human NASH, and its contribution to NASH[84]. Therefore, it is likely that multiple forms of hepatocyte death act as key triggers in NASH[44], but that they may differentially affect cell-cell communication leading to inflammation and fibrogenesis[43,44]. Dead hepatocytes can be removed by efferocytosis by professional phagocytes such as macrophages, triggering TGFβ release; or they can spill their contents in a less controlled fashion as in the case of necrosis, necroptosis and other non-apoptotic forms of cell death, thereby triggering a wide range of signals, in particular inflammation, in resident and non-resident cell types of the liver[43,44]. Cell-cell communication triggered by hepatocyte apoptosis and subsequent efferocytosis remain uncharacterized in NASH fibrosis. One essentially unanswered question is whether efferocytosis contributes to protective and antifibrotic cell-cell communication by removing dead hepatocytes and reducing inflammatory signaling induced via DAMPs from hepatocytes that underwent apoptosis, necroptosis and/or secondary necrosis; or whether it

is part of a fibrosis-promoting response via activation of a TGFβ-secreting macrophage-HSC network (discussed in detail below). Finally, reports demonstrating activation of HSC after their engulfment of apoptotic bodies in vitro[71] and in vivo[72] suggests direct links between hepatocyte death and HSC activation. As efferocytosis can be carried out by epithelial cells, it is also conceivable that hepatocytes have a role in efferocytosis of dead hepatocytes in NASH[85].

Stressed, injured or "undead" hepatocytes as trigger for fibrosis.—Hepatocyte ballooning is one of the most characteristic features of NASH. Ballooning is associated with higher risk for NASH and fibrosis development[86,87] and decreased long-term survival[6]. While ballooned hepatocyte may be on the path to cell death and/or more sensitive to apoptosis, they are generally considered to be injured but living hepatocytes[88,89] and have hence been termed as "undead" hepatocytes [90]. Ballooned hepatocytes secrete sonic hedgehog (SHH), which promotes HSC activation[37], but it is likely that they secrete additional profibrogenic ligands. While above-described associations suggest a key contribution to NASH fibrosis, is it conceivable that ballooned "undead" hepatocytes are a surrogate for hepatocyte stress, implying that hepatocyte stress drives fibrosis development in NASH without the need for hepatocytes to reach a state of ballooning or death. As such, ER stress is induced in steatotic livers from high-fat diet-fed mice[91], and ER stress is common feature of fatty liver and NASH in patients[92]. Hepatocyte ER stress contributes to steatosis, hepatoycte death, inflammation and fibrosis[93], suggesting that long-term and maladaptive ER stress triggers key features of NASH. Moreover, decreased expression of mitofusin 2, observed in NASH patients and mice on high-fat diet, may contribute to NASH by increasing steatosis and fibrosis in an ER stress-dependent manner via reduced transfer of phosphatidylserine from ER to mitochondria[94]. Hepatocyte ER stress also induces caspase 2, which contributes to NASH development[95].

Hepatocyte signaling pathways that promote HSC activation and fibrosis in NASH.

TAZ.—TAZ, a paralogue of YAP and key component of the HIPPO-YAP/TAZ-TEAD signaling cascade, is strongly upregulated in hepatocytes in mouse models and patients with NASH[38]. Interestingly, TAZ upregulation appears to be confined to NASH as there was no increase in TAZ protein expression in CCl_4-induced liver injury[38]. Moreover, TAZ was not upregulated in simple steatosis, suggesting that TAZ could be involved in the transition from simple steatosis to NASH[38]. Indeed, the functional contribution of hepatocyte TAZ to NASH development was proven by hepatocyte-specific deletion and silencing, with therapeutic efficacy even in advanced stages[38,96]. TAZ may affect fibrogenesis in NASH through multiple mechanisms (Fig.2): TAZ silencing reduces the expression of its target Indian Hedgehog (IHH), which exerted profibrogenic effects on HSC in vitro; conversely, NASH-induced fibrosis in vivo was suppressed by IHH silencing[38]. Further, IHH, like TAZ, is increased in human NASH but not simple steatosis[38,97]. Another study reported reduced fibrosis in a NASH-driven HCC model after IHH deletion in hepatocytes[98]. In addition to reducing IHH, TAZ silencing also decreased hepatocyte death and inflammation[38]. Owing to its effects on hepatocyte death, inflammation and HSC activation, hepatocyte TAZ appears to be a key hub in disease-promoting cell-cell communication in NASH and NASH fibrosis. TAZ upregulation occurs predominantly through transcription-independent pathways[38], but

the pathways upregulating TAZ in NASH remain unknown. As TAZ is not upregulated in NASH in simple steatosis or models of toxic liver injury suggest that TAZ increases are not explained by triglycerides or hepatocyte injury but linked to yet undefined factors associated with NASH. Moreover, functional interactions of TAZ with its paralogue YAP as well as other fibrosis-promoting pathways including Notch need to be further explored in order to determine whether there is a general reactivation of developmental pathways in NASH. In this context, a recent study showed a key role for hepatocyte YAP and its target Cyr61 in CCl_4-induced liver fibrosis[99].

Notch.—Notch is a developmental pathway with roles in cell fate decisions[100], contributing to the differentiation of hepatocyte progenitors towards cholangiocytes[101]. Whereas Notch activity is nearly absent in hepatocytes in the healthy adult liver and mildly elevated in simple steatosis [102], it is substantially increased in murine and human NASH[39]. Hepatocyte-specific Notch loss-of-function mouse models showed attenuated NASH-associated liver fibrosis without affecting cell death and inflammation[39], thus distinguishing the effects of Notch on NASH-induced fibrosis from TAZ, which affects all these parameters[38] (Fig.2). Analysis of the secretome of Notch-activated hepatocytes revealed an increase of osteopontin, which was responsible for the majority of the profibrogenic effects of Notch activation (Fig.2), both in vitro and in vivo[39]. Mechanisms of increased Notch activity are not as yet clear, but as ligand availability is normally limiting, the positive association of liver JAG1 expression (but not other Notch ligands)[103] with propensity to NASH/fibrosis is intriguing. Also noteworthy is the finding that Notch activation increases FoxO1 activation at gluconeogenic promoters, leading to glucose intolerance[104], which may partially explain the well-appreciated association between type 2 diabetes and accelerated NASH pathology.

Hh.—The hedgehog (Hh) pathway exerts fundamental morphogenic and mitogenic roles in tissue development, homeostasis, and repair[105]. Hh exerts important roles in hepatic injury responses and fibrogenesis[106]. As described above, human NASH liver, but not steatotic liver, expresses IHH, and causation data in vitro and in mice show a pro-fibrotic role of IHH in NASH[31]. In NASH patients, ballooned hepatocytes express Hh ligand SHH and are surrounded by Gli2-positive, i.e. Hh-activated myofibroblasts. ER stress, a common feature of NASH, results in increased expressed of SHH[37]. These findings suggest that stressed and ballooned hepatocytes generate Hh ligands which act as paracrine pro-fibrogenic factors for Hh-responsive stromal cells. Accordingly, hepatic SHH expression correlates with ballooning, Mallory-Denk bodies, fibrosis, ductular reaction, lymphocytic infiltration and serum AST[107,108]. However, patients with holoprosencephaly, a disorder that is often caused by inactivating mutations of SHH signaling, display increased liver pathology, in particular steatosis[109]. Likewise, Gli2 heterozygosity increases liver steatosis[109]. Together, these findings suggest a dual role of SHH in NAFLD, to suppress hepatic triglyceride accumulation while promoting the development of fibrosis. Whether SHH and IHH exert similar or distinct roles in NASH, remains to be further evaluated. Moreover, it is not clear whether all effects of Hh ligands SHH and IHH are directly on HSC or whether they may also promote HSC activation and fibrosis indirectly.

Crosstalk between hepatocyte TAZ, Notch, Hh and other profibrogenic pathways.: The YAP/TAZ and Notch pathway can interact, either by transcriptional coregulation of common target genes or via the YAP/TAZ-mediated transcriptional upregulation of Notch ligands[110]. For instance, YAP upregulates Notch2 to promote transdifferentiation of hepatocytes towards the cholangiocyte fate[111]. However, interactions between Notch and TAZ, two key drivers of NASH development, remain unexplored in NASH. Notch promotes fibrogenesis without altering liver injury whereas TAZ affects both processes, suggesting distinct mechanisms of action. Further studies are needed to understand whether YAP/TAZ upregulate Notch in NASH and thereby promote fibrosis and whether Hh signaling intersects with YAP/TAZ and/or Notch in NASH. Moreover, further studies are needed to determine whether the activation of these different development pathways is triggered by conserved upstream regulators. Similarly, TAZ, Notch and Hh pathways are thought to promote HSC activation and fibrosis independently of TGFβ, with TAZ and Notch signaling via IHH and osteopontin, respectively. But whether TGFβ signaling is amplified by TAZ-IHH- and Notch-OPN-mediated signals, and whether dual inhibition represents a rationale therapeutic strategy requires further study.

DAMPs.: Although it has been suggested that DAMPs from dying or stressed hepatocytes may promote liver fibrosis[43,44,70], there is currently no convincing evidence to pinpoint a key role of classical DAMPs. As such, recent studies clearly show no effect of HMGB1 deletion on liver fibrosis[112,113]. However, the concept that hepatocyte-released DAMPs act on specific receptors on macrophages or HSC to promote fibrosis directly or indirect. Indeed, we have identified a profibrogenic HSC-enriched DAMP receptor (RFS, unpublished data).

How metabolic alterations trigger NASH-promoting pathways and cellular crosstalk

It is widely accepted that hepatic insulin resistance contributes to the development of NAFLD and that this is at least in part explained by selective insulin resistance in hepatocytes, which lose their ability to suppress glucose production in response to insulin while retaining the capacity to drive lipogenesis and increasing de novo lipogenesis[114]. This may be explained by Notch-driven hepatic insulin resistance[104] and de novo lipogenesis[115], but identification of molecular regulators of this selective insulin resistance paradox has proven elusive. Recent data support a role for the Akt Ser^{473} phosphatase, PHLPP2, as a molecular uncoupler of insulin-mediated repression of gluconeogenesis and activation of lipogenesis. Akt is a critical signaling node in determining insulin action– within minutes [116], Akt phosphorylates FoxO1 to repress glucose production; later, Akt activates mTORC1 signaling by phosphorylation of TSC2[117], leading to increased Srebp1c activity at lipogenic gene promoters. PHLPP2 levels are decreased in obese liver[118]; adenoviral rescue reduces lipogenesis, whereas hepatocyte-specific PHLPP2 deletion is sufficient to cause fatty liver even in mice fed a normal chow diet[119]. Remarkably, PHLPP2 gain- and loss-of-function mice show unchanged glucose homeostasis. These data suggest that insulin signaling is not paradoxically selective in obesity, but rather that downstream elements determine relative effects on glucose production and lipogenesis.

But while this mechanism explains hepatocyte steatosis, it remains elusive which metabolic alterations trigger NASH promoting pathways and why the majority of people with steatosis do not develop NASH. Lipid droplets are often considered an inert storage site that puts lipids out of harm's way, but a strong overload of this protective system could trigger the activation of NASH-promoting pathways such as TAZ, Notch or Hh. Thus, while steatosis is the key defining feature of NAFLD and NASH, it remains uncertain whether it is merely a required element ("first hit") that sensitizes the liver to injury by mediators such as endotoxin or TNF ("second hit") and progression to NASH. Alternatively, lipids may directly contribute to disease progression. Even though the majority of studies have not found a relationship between the degree of steatosis and NASH development[3,6], the loss of steatotic hepatocytes in more advanced stages due to ECM accumulation and "burnt-out" disease confound these analyses. Indeed, recent studies found a positive correlation between the degree of hepatic steatosis, fibrosis development[120] and liver disease mortality[121]. These findings are in line with epidemiologic data showing an influence of single nucleotide polymorphisms in PNPLA3 or TMSF6, two genes with roles in lipid metabolism, on fibrosis development[122,123].

Hepatic diacylglycerol, triacylglycerol, saturated free fatty acids and free cholesterol increase in NAFLD and NASH[124]. It is now believed that triglycerides contribute to steatosis but not to injury and fibrosis[125,126], whereas "bad" fats such as saturated fatty acid [127,128] or free cholesterol[129] trigger lipotoxicity. Consistent with the concept that triglyceride-containing lipid droplets serve as a protective buffer, mice lacking diacylglycerol acyltransferase 2 with inability to convert FFAs into inert intracellular triacylglycerol show increased injury and fibrosis[125]. Mechanisms by which harmful lipids trigger NASH-promoting signaling pathways in hepatocytes - such as TAZ, Notch and Hh - and the subsequent activation of the NASH-promoting multicellular network described above remain elusive (Fig.2). It is conceivable that each NASH-promoting signaling pathway is activated by specific lipids; or that multiple harmful lipids, possibly in conjunction with additional hits, converge, triggering the activation of multiple NASH-promoting signaling pathways in parallel. Accordingly, several key hepatocyte proteins involved in lipid metabolism, including ACC-1/2, FXR/FGF19/FXR4, SCD-1, are promising therapeutic targets that are investigated in clinical trials[5], thus highlighting the key role of hepatocyte lipid metabolism as initiator of the fibrosis-promoting hepatocyte-macrophage-HSC crosstalk in NASH.

In addition to affecting hepatocytes, metabolic changes may affect inflammatory cells and may – in concert with their effects on hepatocytes - contribute to NASH development. Although "metabolic activation" of NKT and T cells in NASH has been suggested [45], no evidence exists that this constitutes a direct metabolic activation independent of hepatocyte steatosis and injury. However, there is evidence supporting a role for lipid-mediated activation of macrophages and HSC. Saturated fatty acids as well as peroxidized lipids induce a proinflammatory macrophage phenotype[130]. In NASH, free cholesterol accumulates in macrophages, some of which is esterified to cholesteryl fatty acid esters, which causes them to resemble foam cell macrophages in atherosclerotic lesions[131]. Moreover, cholesterol crystals within lipid droplets of dead hepatocytes induce the formation of crown-like structures, resulting in macrophage activation with high levels of NLRP3 and

caspase 1[132]. Oxidized LDL is sequestered in Kupffer cells lysosomes when injected into hyperlipidemic mice, resulting induced inflammatory gene expression[133]. In HSC, free cholesterol increases after feeding mice high-cholesterol diets, contributing to HSC activation and fibrosis via increased expression TLR4 or amplification of TLR4 signaling[134–136]. In summary, it appears that cholesterol not only contributes to NASH- and fibrosis-promoting changes in hepatocytes but also in other cell types such as HSC and macrophages. Although the relative contribution in these different cell types remains to be determined, it is likely that hepatocytes are the main target of NASH-promoting lipids.

Macrophages in HSC activation, fibrogenesis and fibrosis resolution in NASH

While the majority of macrophages in the healthy state are liver-resident yolk sac-derived Kupffer cells[137], there is a marked infiltration of bone marrow-derived macrophages in the setting of liver injury, including NASH[130]. scRNA-seq studies have revealed macrophage heterogeneity showing the presence of Kupffer cells, tissue monocytes, and CD9+ TREM2+ NASH-associated macrophages (NAM)/scar-associated macrophages (SAM) in mice and patients[28,138], the latter being expanded in cirrhotic livers and accumulating within fibrotic septa. Liver macrophages contribute to toxic liver fibrosis as demonstrated by pharmacologic and genetic ablation as well as by knockout of mediators of macrophage recruitment[139–142]. Similarly, macrophage depletion or inhibition of macrophage recruitment via CCR2/CCR5 inhibition suppresses fibrogenesis murine NASH[143,144]. TGFβ represents the most potent mediator of HSC activation and fibrosis and is enriched in macrophages[31]. Moreover, SAM express multiple other fibrosis-promoting genes including IL1B, SPP1, PDGFB and TNFSF12[28]. Conversely, HSC/fibroblasts, which are in close proximity to SAM, express cognate receptors that recognize IL-1B, SPP1, PDGFB and TNFSF12, rendering them highly responsive to these SAM-secreted profibrogenic factors and making the SMA-HSC/fibroblast axis an essential contributor to fibrogenesis[28]. As such, macrophage-derived IL-1β may promote the survival of HSC, thus increasing the pool of ECM-producing myofibroblasts[142]; SPP1, encoding osteopontin directly promotes HSC activation[145]; TNFSF12 and PDGFB promote HSC proliferation[28]. It is likely that the SAM-HSC axis acts in concert with other cell types and that for example TGFβ may also derived from cells such as activated HSC[31] and contributes to the “perpetuation” of HSC activation. In NASH, macrophages exert additional functions that may modulate fibrogenesis such as the modulation of hepatic insulin sensitivity, and hepatic inflammation[146] (discussed below).

Liver macrophages not only contribute to HSC activation and fibrosis but also to ECM degradation during the regression stage. CD11b+F4/80+Ly6C^{hi} (mice) or CD14+/CD16+/CCR2+ (human) macrophage populations[147] are fibrogenic, contrasting the murine CD11b^{+} F4/80^{+} Ly-6C^{lo} population of restorative macrophages (the human restorative macrophage phenotype is not well-defined) that contributes to ECM degradation[148]. Accordingly, infusion of bone marrow-derived macrophages into mice increased hepatic recruitment of endogenous macrophages and neutrophils, thereby upregulating levels of MMP9, MMP13 and IL-10 in the scar, resulting in improved murine liver fibrosis[149]. Similar restorative functions can be seen in patients, as evidence by a recent trial in which infusion of macrophages was not only safe but also let to a decrease in MELD score in some participants[150]. Even though it appears that profibrogenic macrophages can be converted to

pro-resolution macrophages in the liver[148,151], the signals that regulate the switch to the resolving phenotype are currently not understood.

Macrophages linking hepatocyte damage to HSC activation in NASH via efferocytosis.

Even though saturated fatty acids, cholesterol, and oxidized lipids may directly trigger macrophage activation in NASH, it appears that NASH-induced hepatocyte damage and cell death are the dominant drivers of progression and fibrosis[43,44], triggering macrophage recruitment, activation and subsequent macrophage-mediated HSC activation. One of the main purported functions of macrophages is the clearance of dead cells[152]. While apoptotic cell death is considered non-reactive, there is a wide body of literature linking NASH to hepatocyte apoptosis, and hepatocyte apoptosis to fibrosis[44]. Interestingly, the non-inflammatory nature of apoptosis is promoted by active suppression of inflammation and activation of inflammation resolution and possibly fibrogenesis-promoting pathways following the engulfment of apoptotic cells via efferocytosis. Apoptotic cells trigger the recruitment of macrophages through the release of soluble “find me” signals such as ATP (recognized by P2Y2 receptor) , CX3CL1 (recognized by CX3CR1) and sphingosine-1-phosphate (recognized by multiple sphingosine-1-phosphate receptors)[152,153]. Specific “find me” signals released by apoptotic hepatocytes in NASH are currently not known. The subsequent engulfment of apoptotic cells by “eat me” signals is largely triggered by the presence of phosphatidylserine on the outer leaflet of apoptotic cell membranes, which either directly binds to receptors on macrophages such as TIM-4 and αvβ5 integrin or engage with proteins such as GAS6 and PROS1, which in turn engage and activate receptors of the TAM family receptors on macrophages, such as MerTK[152,153]. In addition to the removal of apoptotic cells – which per se is anti-inflammatory - efferocytosis actively suppresses inflammation via the secretion of anti-inflammatory cytokines such as TGFβ[152]. Through this TGFβ pathway, efferocytosis may not only blunt inflammation but also promote HSC activation and fibrogenesis. This could be interpreted as attempt to resolve inflammation while repairing tissue injury in the setting of apoptotic liver injury (Fig.3). There is currently no literature that has experimentally tested the role of efferocytosis and efferocytosis-induced TGFβ in the liver even though one study demonstrated the presence of hepatic macrophages containing annexin V, suggesting macrophage phagocytosis of apoptotic fat-laden hepatocytes[154]. However, it is also conceivable that efferocytosis is a protective response in NAFLD which removes dead hepatocytes, thereby preventing the release of DAMPs and subsequent DAMP-mediated inflammation and fibrosis. If this were the case, failure to efficiently efferocytose and/or a switch from apoptotic cell death to more inflammatory cell death modes such as necroptosis, necrosis or ferroptosis might contribute to disease progression and fibrosis in NASH. It is also possible that efferocytosis of apoptotic hepatocytes – providing profibrogenic TGFβ – and non-apoptotic cell death – resulting in disease-promoting inflammation - exist in parallel and cooperatively promote the development of liver fibrosis. If efferocytosis turns out to be pro-fibrotic, it may be necessary to target multiple receptors to achieve significant therapeutic effects in NASH as each phase of efferocytosis employs multiple and often redundant receptors. It will also be important to understand whether other pathways in hepatocytes, such as the activation of TAZ and Notch but also specific metabolic alterations or ballooning, cooperate with or modulate efferocytosis or efferocytosis-mediated induction of macrophage-derived TGFβ.

Macrophages as instigators of inflammation and metabolic alterations in NASH

Besides directly affecting HSC and fibrosis, macrophages also regulate hepatic inflammation and metabolism, which may indirectly contribute to fibrogenesis. Hepatic macrophages have an important role in regulating inflammation in NASH. Liver macrophages express high levels of TLR4, making them highly responsive to gut-derived LPS, which is increased in NAFLD and NASH[11,155], and contributing to high production of TNF and IL-1β and promotion of inflammation by macrophages in NAFLD[130]. Macrophage YAP contributes to hepatic inflammation but not steatosis or fibrosis in a HFD model of NASH[156]. The lacking effects of macrophage YAP on fibrosis in this study were attributed to low fibrosis induction by this model[156]. In addition, macrophage TNF and IL-1β may enhance fibrosis by directly promoting HSC activation and/or survival[142,157]. Moreover, macrophage-derived inflammatory mediators may also influence hepatocyte steatosis[157,158]. As such, macrophage-derived IL-1β downregulates PPARα, thereby leading to reduced fatty acid oxidation and increased triglyceride accumulation in hepatocytes[158]. Accordingly, macrophage depletion decreased steatosis and hepatic insulin resistance[158,159]. In conclusion, the regulation of hepatic inflammation, steatosis, hepatocyte death, and fibrosis by macrophage-derived cytokines are likely intimately linked and act in concert to drive NASH progression.

HSC effects on other cell types regulating liver injury, inflammation, regeneration, metabolism and liver function in NASH

While HSC are well-characterized as executors of fibrogenesis, it is not known how HSC affect other aspects of NASH. It is possible that the initial role of HSC in NAFLD is restorative via signals to other liver cells, in particular hepatocytes. As such, HSC are a main source of hepatocyte growth factor (HGF). Although the role HSC-derived HGF has not yet been studied, its receptor Met exerts an essential role in hepatocyte regeneration and survival in NASH and other diseases[160–162]. HSC-derived collagen promotes hepatocyte survival via Erk[163] and possibly via integrin- and mechanosensitive signaling pathways. Lastly, it is not known whether HSC and HSC-derived ECM affect hepatocyte function and metabolism in NASH. Consistent with their close proximity to endothelial and hepatocytes, HSC could promote or inhibit hepatic steatosis by affecting inhibiting lipid shuttling into or out of hepatocytes.

Targeting cell-cell crosstalk in NASH

Given the key role of cell-cell crosstalk in NASH and the difficulty to non-selectively inhibit key fibrogenic pathways such as TGFβ due to severe side effects, targeting key intercellular pathways that trigger or maintain these disease-promoting interactions in may be a promising treatment strategy for NASH fibrosis (Fig.4). Hepatocytes represent a key cell driver initating NASH progression and can nowadays be targeted in a cell-specific manner by GalNAc-coupled siRNA, which has recently been approved by the FDA for TTR amyloidosis[164]. This approach can be applied to many hepatocyte pathways that trigger NASH fibrosis. Hepatocyte-specific silencing of TAZ may be useful as prevention as well as treatment as shown by GalNAc-siRNA silencing of TAZ in murine NASH fibrosis in mice[96] and would interrupt several arms of the multi-cellular network that promotes NASH fibrosis,

including hepatocyte death, inflammation and HSC activation (Fig.4). Targeting hepatocyte Notch may also be promising for NASH fibrosis but would require delivery of hepatocyte-targeted Notch inhibitors (Fig.4). In contrast, select targeting of pathways that mediate hepatocyte-HSC crosstalk such as SHH, IHH or osteopontin is likely to only achieve partial inhibition of NASH fibrosis due to the moderate profibrogenic potency of each pathway. Targeting key pathways that contribute to hepatocyte metabolic stress or death via inhibition of ACC, ASK-1, SCD-1 or via activation of FXR, FGF19, PPARα/δ or PPARα/γ signaling may indirectly inhibit the NASH fibrosis-promoting crosstalk between hepatocytes, macrophages and HSC (Fig.4; reviewed in[5]). Macrophages and efferocytosis pathways seem attractive targets as source and potential driver, respectively of TGFβ release in NASH fibrosis. However, we first need to learn more about the role of efferocytosis in NASH as it might also exert protective effects. Therefore, inhibition of macrophage recruitment by blockade of chemokine receptors such as CCR2 and CCR5 (Fig.4) seems a more feasible strategy to block cell-cell communication at the level of macrophages. CCR2/5 antagonist Cenicriviroc improved macrophage recruitment, steatosis, NAS score and fibrosis in mouse models of NASH[144,165] and increased the percentage of patients who had improvement in fibrosis by ≥1 stage[166]. Currently, there are no approaches to specifically target macrophage-HSC crosstalk in NASH. However, given that liver macrophage-selective silencing via nanoparticles is feasible[167], one could also envision macrophage-specific silencing of TGFβ as therapy for NASH fibrosis (Fig.4), although this strategy may increase liver inflammation. Infusion of macrophages is another possibility that may activate cell-cell networks leading to increased MMP and IL-10 expression and subsequent decreases in fibrosis and is currently tested in patients with cirrhosis of various etiologies[150].Targeting of HSC is likely useful for directly inhibiting fibrogenesis in this cell type, rather than inhibiting cell-cell crosstalk in NASH, and is currently tested using a vitamin A-coupled lipid nanoparticle containing siRNA against HSP47, which leads to collagen misfolding and HSC death (NCT02227459). Finally, anti-platelet therapy using ticragelor or aspirin+clopidogrel may be beneficial in NASH, interrupting the crosstalk with hepatocytes and immune cells, thereby decreasing steatosis, inflammation and injury[49]. In summary, direct targeting of cell-cell communication or indirect targeting – via upstream triggers within hepatocytes that initiate this NASH-promoting cell-cell crosstalk - appear to be promising strategies for treating NASH fibrosis. However, further efforts are needed to establish the safest and most potent approaches.

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Biographies

Abbreviations:

NAFLD	non-alcoholic fatty liver disease (NAFLD)
NASH	non-alcoholic steatohepatitis
HSC	hepatic stellate cell
ECM	extracellular matrix
TGFβ	transforming growth factor-β
IHH	Indian hedgehog
SHH	sonic hedgehog
Hh	hedgehog
LSEC	liver sinusoidal endothelial cells
BDL	bile duct ligation

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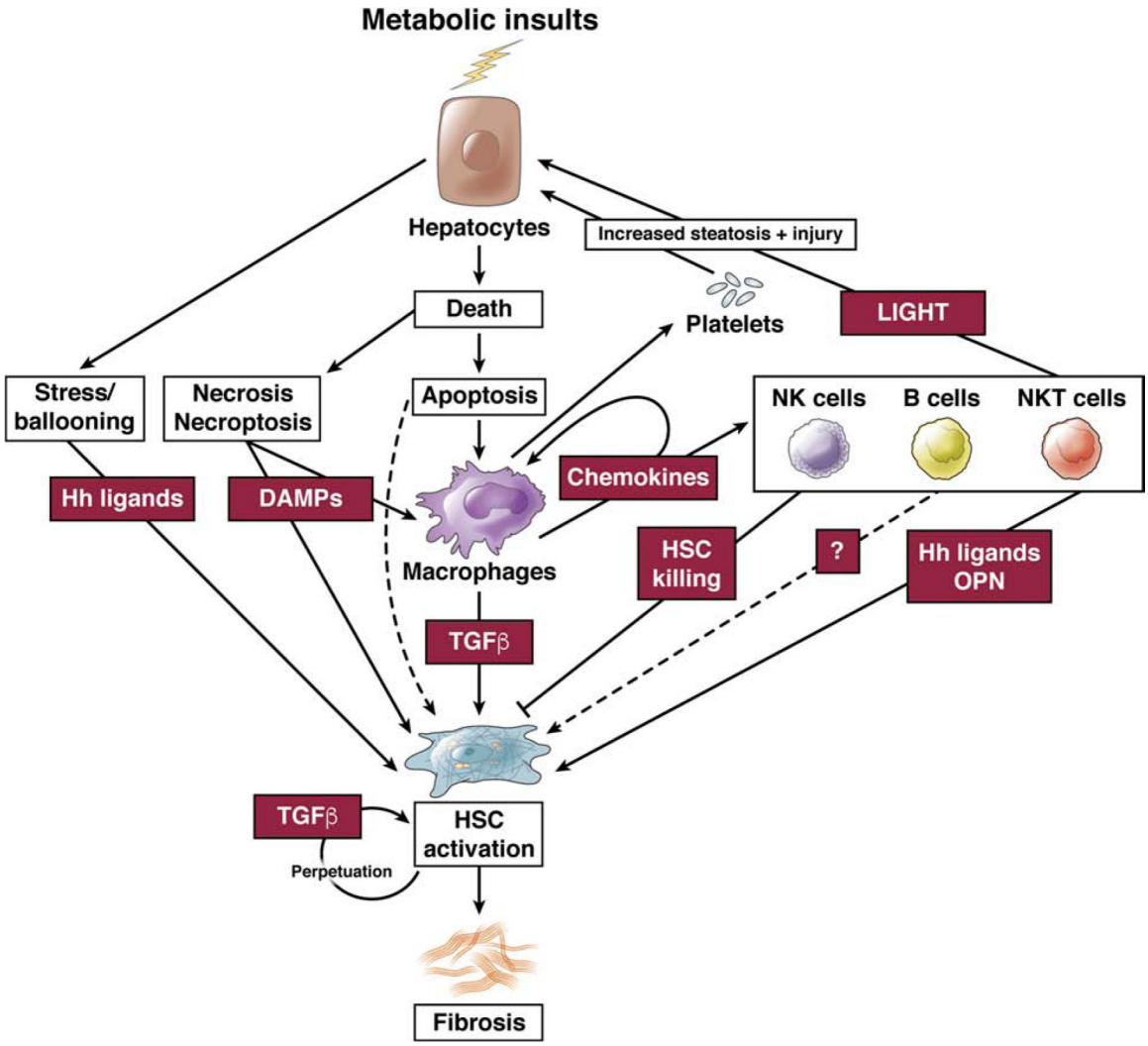

Fig.1. The cellular network regulating HSC activation and fibrosis in NASH.

Metabolic insults promote hepatocyte steatosis and injury, activating a multi-cellular network consisting of macrophages, NKT cells, NK cells, B cells and NK cells that control HSC activation and the development of fibrosis.

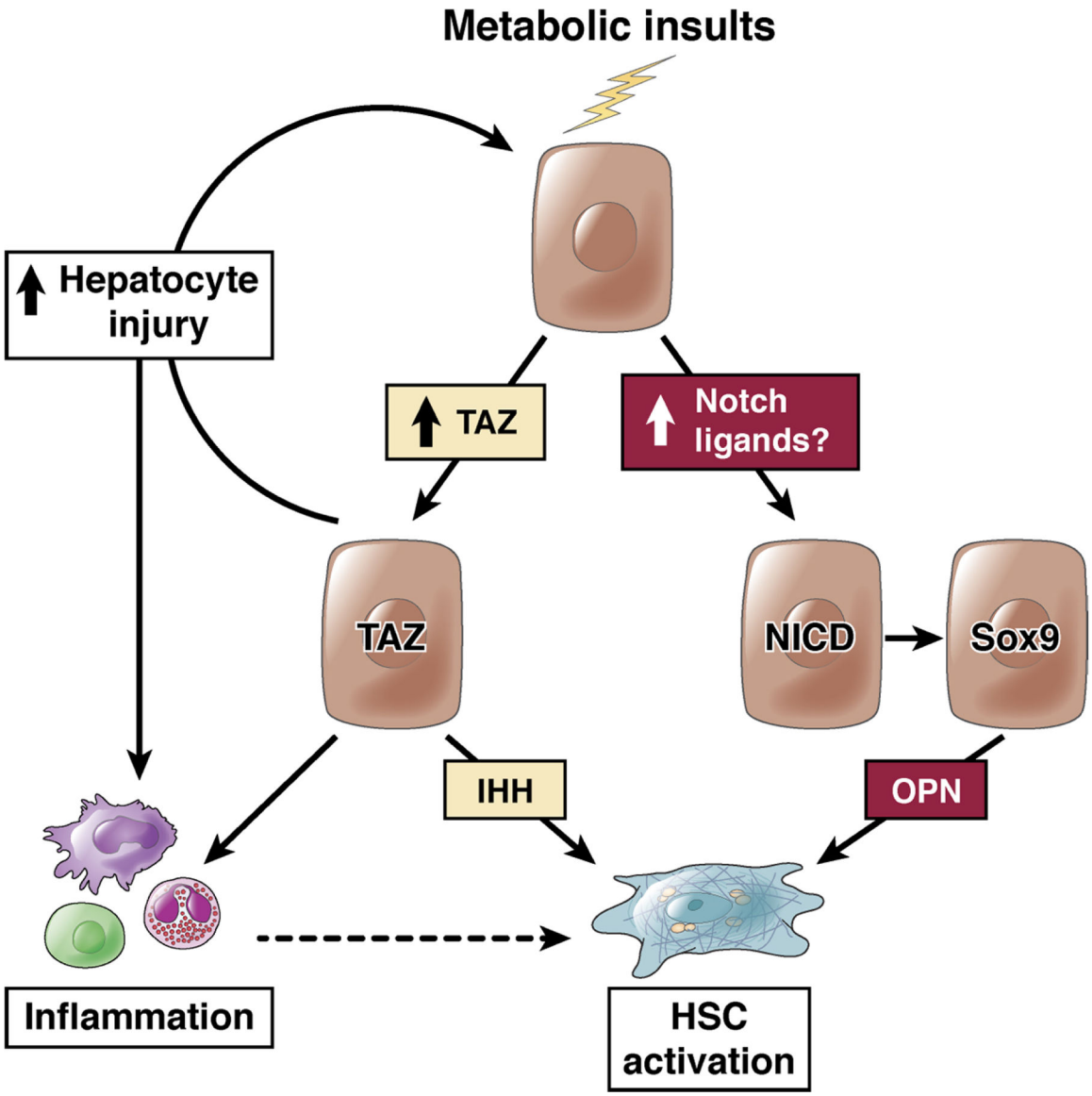

Fig.2. The role of hepatocyte TAZ and Notch in NASH fibrosis.

Metabolic insults lead to the activation of TAZ and Notch in hepatocytes. Increased hepatocytes expression of TAZ in NASH (but not simple steatosis) directly leads to HSC activation via the release of IHH, and additionally promotes hepatocyte injury and inflammation, which may indirectly promote HSC activation. Notch activity, driven by cell-surface ligands on a neighboring cell, leads to Sox9-dependent increase in osteopontin secretion, to activate HSC.

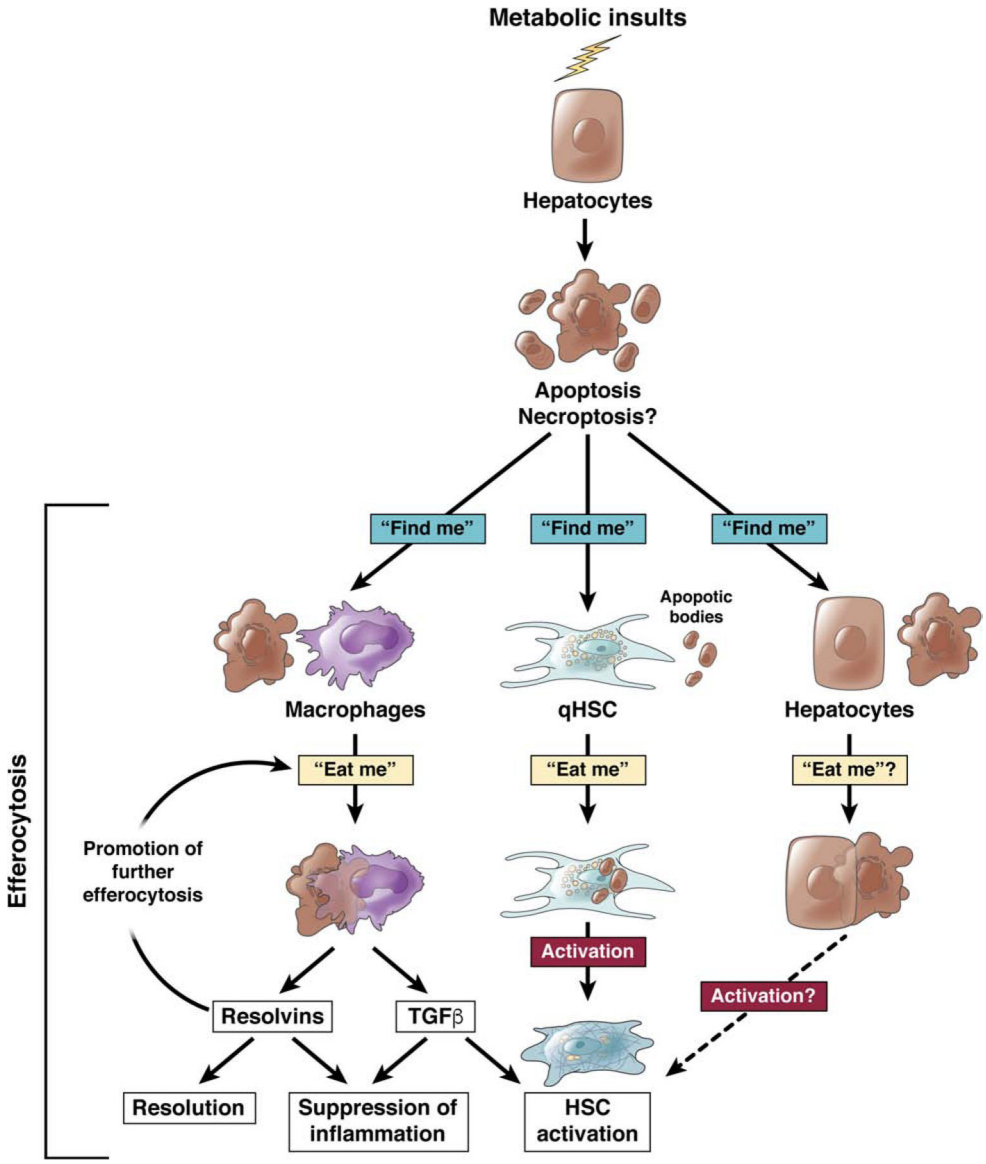

Fig.3. Efferocytosis of dead hepatocytes as promoter of HSC activation and fibrosis.

Apoptotic hepatocytes may be detected and engulfed by macrophages in response to "find me" and "eat me" signal in a process termed efferocytosis. Efferocytosis is most commonly exerted by professional phagocytes such as macrophages, where it leads to the release of resolvins – suppressing inflammation and promoting resolution - and TGFβ - suppressing inflammation and promoting HSC activation. Efferocytosis has also been suggested to occur in HSC and promote their activation. It is conceivable that efferocytosis could occur in hepatocytes, directly or indirectly affecting HSC activation.

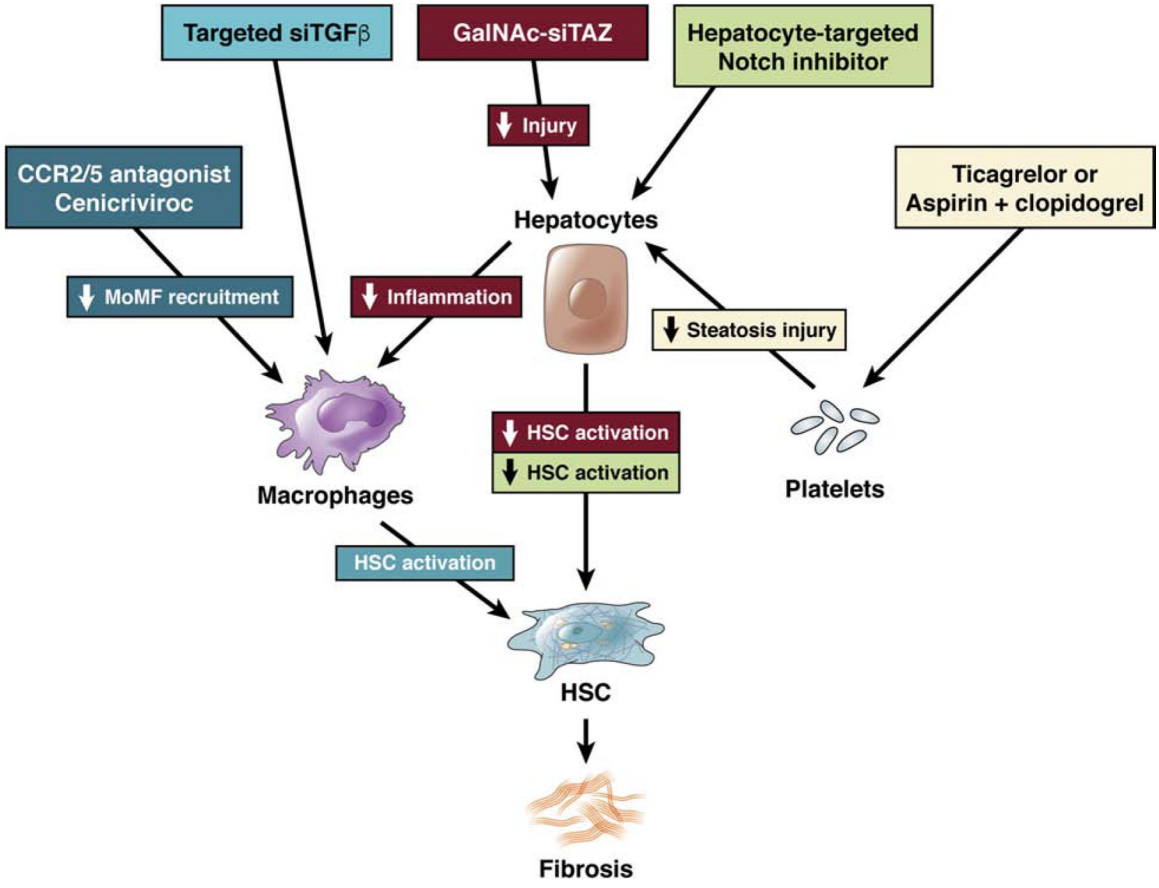

Fig.4. Therapeutic inhibition of NASH by targeting intercellular networks.

Targeting metabolic pathways, either in hepatocytes (ACC, SCD1, FXR/FGF19) or upstream (PPARα/δ or PPARα/γ) will improve hepatocyte metabolism and health and thereby indirectly reduce the fibrosis-promoting crosstalk with macrophages and HSC. Pathways that more directly initiate fibrosis-promoting crosstalk may be targeted at different levels.. Hepatocyte-specific TAZ silencing via GalNAc-coupled siRNA may lead to a reduction of IHH-mediated HSC activation as well as reduced inflammation and hepatocyte injury in NASH. Hepatocyte-targeted Notch inhibitors may decrease HSC activation in NASH fibrosis. Targeting the recruitment of monocyte-derived macrophages (MoMF) via CCR2/5 antagonist Cenicriviroc or the release of TGFβ from macrophages may reduce HSC activation in NASH. Targeting of the platelet-hepatocyte crosstalk via anti-platelet therapies such as Ticagrelor or aspirin+clopidogrel may reduce hepatocyte steatosis and injury, and a subsequent secondary reduction of HSC activation in NASH.