Understanding the cellular interactome of non-alcoholic fatty liver disease

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Summary
Non-alcoholic fatty liver disease (NAFLD) is reaching epidemic proportions, with a global prevalence of 25% in the adult population. Non-alcoholic steatohepatitis (NASH), which can lead to cirrhosis, has become the leading indication for liver transplantation in both Europe and the USA. Liver fibrosis is the consequence of sustained, iterative liver injury, and the main determinant of outcomes in NASH. The liver possesses remarkable inherent plasticity, and liver fibrosis can regress when the injurious agent is removed, thus providing opportunities to alter long-term outcomes through therapeutic interventions. Although hepatocyte injury is a key driver of NASH, multiple other cell lineages within the hepatic fibrotic niche play major roles in the perpetuation of inflammation, mesenchymal cell activation, extracellular matrix accumulation as well as fibrosis resolution. The constituents of this cellular interactome, and how the various subpopulations within the fibrotic niche interact to drive fibrogenesis is an area of active research. Important cellular components of the fibrotic niche include endothelial cells, macrophages, passing immune cell populations and myofibroblasts. In this review, we will describe how rapidly evolving technologies such as single-cell genomics, spatial transcriptomics and single-cell ligand-receptor analyses are transforming our understanding of the cellular interactome in NAFLD/NASH, and how this new, high-resolution information is being leveraged to develop rational new therapies for patients with NASH.

Introduction
Chronic liver disease (CLD) and mortality from end-stage liver failure are increasing globally and have become critical public health challenges of our time. Worldwide, CLD and its complications cause nearly 2 million deaths, i.e. more than 1/30th of all-cause mortality.1 Europe has the highest relative death rate related to CLD.2 In the UK, cirrhosis-related complications, including hepatocellular carcinoma, are the fastest growing cause of preventable death, with a 400% increase in standardised mortality since 1970.3 In the US, CLD and cirrhosis are the 12th leading cause of death in all age groups, but the fourth leading cause of death in the 45–64 years age group.4

The global epidemic of obesity, diabetes and the metabolic syndrome has been mirrored by an increase in the number of patients with non-alcoholic fatty liver disease (NAFLD), with an estimated global prevalence of 25%.5 Non-alcoholic steatohepatitis (NASH), the progressive form of NAFLD associated with inflammation, fibrosis and increased liver-related mortality, affects up to 30% of all patients with NAFLD.6 NAFLD is becoming the number one indication for liver transplantation in Europe and the USA.2,5

Cirrhosis is the common endpoint of liver fibrosis caused by any iterative liver injury. It is characterised by progressive fibrosis of the liver parenchyma with disruption of hepatic architecture, aberrant regeneration, abnormal vasculature and ultimately, loss of liver function.7 At the histological level, cirrhosis demonstrates major architectural disruption, with regenerative nodules separated by fibrous septa.8 Early NASH fibrosis is thought to begin around the central vein and in perisinusoidal regions, with progression to more generalised, portal-portal bridging fibrosis, as seen in advanced cirrhosis of all aetiologies. Liver fibrosis can both progress and regress, displaying remarkable inherent plasticity and reversibility if the underlying injurious agent is removed. The natural history of NASH is less predictable than other aetiologies of cirrhosis, such as hepatitis C, where progression of fibrosis closely correlates with the underlying disease activity and resolution can lead to significant reversal of fibrosis. Steatosis does not define the degree of fibrosis nor the progression of the disease, but reversal of fibrosis is often observed with weight loss of >10% in obese patients with NAFLD.9,10

Keywords: Non-alcoholic fatty liver disease (NAFLD); cellular interactome; single-cell genomics; anti-fibrotic therapies; fibrosis; cirrhosis
In contrast to many other human chronic liver diseases, NASH displays a unique pathogenesis as it forms part of the metabolic syndrome, a multi-system disturbance of metabolic regulation. Furthermore, the iterative, unresolved inflammation observed in NASH creates favourable conditions for fibrosis which are self-perpetuating and indeed become amplified by intrahepatic and extrahepatic factors such as cellular senescence, hepatocyte dedifferentiation (with concomitant increases in ductular reaction and progenitor cells), as well as adipose tissue inflammation and gut-liver axis-driven inflammation. Lipotoxicity causes hepatocyte stress and cell death via multiple mechanisms including altered mitochondrial function, endoplasmic reticulum (ER) stress, the activation of death receptors and inflammatory signalling cascades, as well as increased oxidative stress. This is often compounded by oxidative stress from hyperglycaemia when diabetes is present. When these ‘multiple-hits’ reach a yet ill-defined threshold of hepatocellular stress and injury, they activate multicellular circuits involving liver-resident cells, including hepatic stellate cells (HSCs), Kupffer cells (KCs, i.e. liver-resident phagocytes) and endothelial cells, as well as a wide range of bone marrow-derived myeloid and lymphocytic immune cells. To date, it has proven challenging to develop effective therapeutic strategies for NASH fibrosis that target a singular cell type or pathogenic mechanism, which emphasises the importance of a comprehensive understanding of cell-cell interactions in the inflammatory and pro-fibrotic microenvironment. Future effective treatments may require combined targeting of several pathomechanisms simultaneously, including pathological cell interactions in the fibrotic niche. Furthermore, in progressive NASH, the topography of the liver micro-architecture includes areas of regeneration alongside areas of unresolved inflammation and fibrogenesis, with stage-specific compositions and interactions in these fibrotic niches. This review will describe how rapidly evolving technologies such as single-cell genomics are transforming our understanding of the multicellular pro-fibrotic interactome in NASH, and how deep insights into the pathogenic mechanisms of human NASH at the cellular and molecular level are being leveraged to develop rational new therapies for patients with NASH.

**Determining cell-cell interactions in NASH**

Given the importance of cell-cell interactions for all aspects of NASH, there have been many efforts to study these interactions in vitro and in vivo. Co-culturing cells using conventional 2D methods or in 3D spheroids allows for functional characterisation of these interactions, but is hampered by the poor reproduction of the environment that drives NASH in vivo, with investigations often limited to 2 subpopulations rather than the multicellular environment. Furthermore, these culture systems, in addition to precision-cut liver slice systems, lack flow conditions, thereby hampering the understanding of extrahepatic mediators (e.g. nutritional products or microbial-associated molecular patterns from the gut, adipose tissue mediators) and patrolling immune cells. Some recently engineered perfused liver-on-a-chip models try to overcome these limitations but can only partially mirror the natural cell organisation in the fatty liver. Until recently, interactions between cell types in vivo could only be assessed one at a time through immunohistochemistry and pharmacologic or genetic inhibition studies. With the advent of single-cell RNA-sequencing (scRNAseq), methods have been developed to determine the interactions between cell types based on the expression of ligand-receptor pairs and repositories for these interactions. Despite some dropout inherent to scRNAseq, a significant percentage of these mRNAs are captured in every cell, allowing for nearly genome-wide and largely unbiased analysis of interactions between all cell types within a given tissue, both in normal and diseased conditions. Multiple computational tools and associated ligand-receptor interaction databases have been developed to elucidate these interactions, with platforms such as CellPhoneDB, CellChat and ICCNET taking into account that interactions often occur between heterodimers or multi-subunit receptors and ligands. However, the spatial context of these interactions, which are often local, has not been sufficiently integrated into these platforms. Integration of spatial transcriptomics into these platforms is considered a next step to more reliably assess cell-cell communication. Likewise transcriptomics may not accurately reflect protein expression and does not accurately capture many metabolites as well as other biologically active ligands, such as lipids or bile acids, that are not directly encoded by specific mRNAs, thus requiring integration of proteomics or other advanced multimodal analyses in the future. CellPhoneDB analysis has revealed numerous cell-cell interactions in mouse models of NASH and in patients with cirrhosis, including NASH-associated cirrhosis, and has revealed mesenchymal cells, macrophages and endothelial cells as having intense interactions with other cell types. However, dedicated analyses of these interactions in different stages of human NASH will be needed to improve our understanding and targeting of NASH. Newly developed platforms such as multicellular organoids and liver-on-a-chip microfluidic systems can be used to further study these interactions functionally and evaluate therapeutic strategies that target specific ligand-receptor interactions.  

**Hepatocytes and their interactions**

The pro-fibrotic microenvironment in NASH involves complex interactions of parenchymal and non-parenchymal cells which drive and perpetuate NASH progression. Pathogenesis can be viewed from the starting point of hepatocyte injury. Caloric overload, steatosis, oxidative stress, ER stress and hepatocyte ‘lipopoiesis’ represent first steps in cell-cell communication,
trigerring the fibroinflamatory cycle.\textsuperscript{13,23} Hepatocytes are the major regulators of lipid metabolism, mediating the conversion of lipids to stable, non-toxic, macrovesicular fat, which may act as a buffer to prevent hepatocyte injury. Excess lipids cause direct toxicity to hepatocytes via oxidative stress, whereby fatty acid oxidation generates reactive oxygen species that deplete antioxidant reserves.\textsuperscript{24} Accumulation of fatty acids leads to the collapse of the mitochondrial membrane potential, cessation of electron transport and subsequent activation of pro-apoptotic signalling via factors such as tumour necrosis factor (TNF)\textsuperscript{a} and NF-\kappaB (the latter having both pro and anti-apoptotic potential).\textsuperscript{25,26} Fatty acids also directly interact with transcription factors and receptors, such as hepatocyte nuclear factor 4 \(\alpha\) (HNF4\(\alpha\), which regulates metabolism and hepatocyte identity, and toll-like receptors (TLRs), which regulate the immune response, contributing to an altered and pro-inflammatory environment.\textsuperscript{25,27} In parallel, cholesterol and other mediators contribute to changes in the transcriptional programmes in steatotic hepatocytes, leading to an upregulation of developmental pathways that are often seen in bipotential progenitor cells or ductular cells, such as Notch and TAZ, which can directly activate pro-fibrotic cell-cell communication between hepatocytes and neighbouring cells.\textsuperscript{28–30}

Hepatocyte injury, apoptosis and necrosis promote a sterile inflammatory response. This includes activation of KCs to clear necrotic debris, recruitment of lymphocytes and bone-marrow-derived monocytes, extracellular matrix (ECM) remodelling, myofibroblast activation, angiogenesis, and hepatocyte regeneration and differentiation to replace lost hepatocytes.\textsuperscript{11,31,32} In NASH, hepatocytes undergo apoptosis, necroptosis or pyroptosis (a newly described caspase I-dependent apoptosis observed in NASH),\textsuperscript{33} thereby initiating inflammation in several ways. They release damage-associated molecular patterns (DAMPs), such as DNA fragments, histones, ATP, uric acid and cholesterol crystals, that act as stress signals to activate DAMP receptors, such as TLRs, P2X and P2Y purinoreceptors and C-type lectin domain (CLEC)12A, which activate innate immunity and inflammation, and often exacerbate tissue injury.\textsuperscript{34} They also secrete inflammatory and fibrogenic cytokines either directly, such as interleukin (IL)-1\(\beta\) and IL-18,\textsuperscript{35,36} or via extracellular vesicles containing C-X-C motif chemokine ligand (CXCL)10, a ligand for C-X-C motif chemokine receptor (CXCR)3, and mitochondrial DNA, a key TLR9 ligand.\textsuperscript{37} Hedgehog pathway activation is triggered by injured, ballooned and/or TAZ-expressing hepatocytes in NASH via the release of sonic hedgehog or Indian hedgehog.\textsuperscript{38,39} In addition to regulating liver regeneration, hedgehog ligands, via their receptor Patched, promote the activation of HSCs in vitro and in mice, and show a positive correlation with progression from steatosis to steatohepatitis and myofibroblast activation in human livers.\textsuperscript{30,39} Likewise, activation of Notch in steatotic hepatocytes contributes to the secretion of osteopontin, which in turn contributes to HSC activation.\textsuperscript{30}

Apoptotic hepatocytes further interact with immune and mesenchymal cells as they undergo the process of effecoryctosis by phagocytes. Whilst this process has anti-inflammatory potential, as it removes intracellular content which could otherwise act as DAMPs, effecoryctosis also triggers macrophages to release transforming growth factor (TGF)\(\beta\). This signalling pathway positively and negatively modulates inflammation in a number of ways, including via the activation of HSCs, a key source of scar-producing myofibroblasts.\textsuperscript{33,41} Apoptotic bodies from hepatocytes can also be engulfed by HSCs and there is evidence that this process leads to the transition of HSCs into myofibroblasts, potentially establishing a direct connection between hepatocyte injury and scar formation.\textsuperscript{42}

Finally, hepatocyte senescence appears to be important in preventing resolution of inflammation, promoting steatosis and driving fibrosis, as elegantly shown by genetic depletion of p16ink4a-expressing senescent cells.\textsuperscript{43} In response to oxidative stress, hepatocytes undergo changes including telomere shortening, nuclear enlargement and damage to genomic and mitochondrial DNA that lead to a senescence-associated secretory phenotype (SASP).\textsuperscript{44} Initially, hepatocyte senescence may be protective, preventing injured cells from proliferating. However, driven by signalling pathways such as NOTCH1, SASP cells contribute to a pro-inflammatory microenvironment by secreting inflammatory cytokines, growth factors and matrix-degrading enzymes which have been implicated in the progression of NASH.\textsuperscript{45,46} In particular, the macrophage chemokines C-C motif chemokine ligand (CCL)2 and TGF\(\beta\) may promote a SASP in neighbouring cells.\textsuperscript{47}

There are several recent studies that revealed disease-driving hepatocyte interactions by single-cell analyses. Wang \textit{et al.} identified a number of potential NASH-upregulated signalling axes, such as TNFRSF11B-TNFFSF10, between hepatocytes and activated HSCs.\textsuperscript{16} Mederacke \textit{et al.} uncovered the purinergic receptor P2Y14 as highly enriched on HSCs and showed that the P2Y14 ligands UDP-glucose, UDP-galactose and UDP-glucuronic acid act as danger signals that are released upon liver injury and promote HSC activation and liver fibrosis.\textsuperscript{48}

**Ductular cells and their interactions**

The unresolved injury and loss of hepatocytes leads to perturbed regeneration and the appearance of a “ductular reaction” in advanced liver diseases including NASH. Cytokeratin (CK)7- and CK19-expressing ductular cells are significant promoters of fibrosis and interact with multiple cell types, including HSCs, portal fibroblasts and a wide range of immune cells.\textsuperscript{49} The interaction between the portal mesenchymal and ductular cells is bidirectional and not only controls the ductular proliferative state but also promotes mesenchymal proliferation. Ductular proliferation precedes mesenchymal expansion and is triggered by soluble factors. Of note, mesenchymal-ductular interactions inhibit ductular proliferation during recovery, mediated by cell contact-dependent signals such as Notch2.\textsuperscript{50,51} Moreover, ductular cells express an array of fibrogenic mediators that have been shown to be upregulated in the human fibrotic liver.\textsuperscript{32,52} The most notable of these are platelet-derived growth factor (PDGF) BB, TGF\(\beta\)1 and TGF\(\beta\)2, and sonic hedgehog, all of which promote myofibroblast activation.\textsuperscript{34–36} In particular, TGF\(\beta\)2 has been identified as a key promoter of progenitor/cholangiocyte differentiation from the peripoal-portal ductular region in regenerating human livers post-transplant. This 'ductular reaction' is perpetuated by pro-inflammatory myofibroblasts which express survival factors to maintain fibrogenic portal-duetal tract formation.\textsuperscript{57} Targeting the pathological interaction between ductal cells and myofibroblasts has shown some therapeutic promise. Integrin \(\alpha v \beta 6\) is a receptor for the ECM proteins fibronectin and tenascin C and can also activate the potent profibrogenic cytokine TGF\(\beta\)1. Integrin \(\alpha v \beta 6\) is highly expressed by ductal cells.\textsuperscript{52} Small molecule inhibition and antibody blockade of integrin \(\alpha v \beta 6\) have both been shown to effectively attenuate biliary and non-biliary fibrogenesis with reduced proliferation of
cholangiocyte-like progenitor cells and attenuated TGFβ1 activation. Most of the studies on mesenchymal-ductular interactions and therapeutic concepts were established in non-NASH settings and therefore need to be further investigated in NASH models.

Single-cell transcriptomic analysis of NASH allowed for a broader and higher resolution interrogation of ligand-receptor interactions between hepatocytes or ductular cells and their cognate signalling partners (Fig. 1). Single-cell-based studies on the interactions between cholangiocytes and mesenchymal cells are emerging, but specific ligand-receptor pairs for these potentially relevant cellular interactions in NASH are still missing.

Kupffer cells and their interactions
Innate immune cells are central regulators of tissue injury and repair. Of these, KCs and bone marrow-derived macrophages (BMDMs, also termed monocyte-derived macrophages) play key roles in both sensing metabolic injury and disturbed tissue homeostasis in NAFLD as well as propagating fibrogenesis and fibrosis. KCs are the resident liver macrophages and in the context of sterile inflammation, remove cellular debris and ECM to facilitate regeneration of healthy tissue. Their dysregulation in NASH can increase chronic inflammation and fibrosis through the production of cytokines such as TGFβ1 and IL-6. Both KCs and BMDMs are activated in acute injury by DAMPs through TLRs and Nod like receptors (NLRs), as well as by inflammatory cytokines. These processes are significantly upregulated in NASH, with KCs recruiting large amounts of Ly6C<sup>hi</sup> blood monocytes that rapidly differentiate into highly phagocytic macrophages in mouse models. Activation of TLR4 on KCs promotes NF-κB signalling, causing further amplification of the inflammatory response, including secretion of cytokines such as TNF-α and CCL2. Concurrently, NLRP3 activation in KCs promotes IL-1β, a critical pro-inflammatory cytokine which induces several components of the acute phase response. Fatty acids can also activate innate immune cells directly via TLR2 and TLR4. These activated phagocytes also produce reactive oxygen species and nitrous oxide which perpetuates macrophage-mediated tissue injury. Pro-fibrotic macrophages coordinate inflammation and fibrosis through a range of interactions with myofibroblasts, for example, amphiregulin, produced by macrophages, activates the integrin-αv-TGFβ axis and induces the differentiation of mesenchymal cells into myofibroblasts.

Bone marrow-derived macrophages and their interactions
Increased recruitment of BMDMs is a crucial event in NASH and during the chronic phase of inflammation, KCs and BMDMs take on differing phenotypes. During self-limiting acute hepatic injury, and after cessation of the acute inflammatory response, macrophages can adopt a phenotype that promotes resolution and tissue remodelling. This pro-resolution macrophage phenotype drives ECM degradation, with upregulation of matrix metalloproteinase (MMP) expression and downregulation of the inflammatory transcriptome, including decreased expression

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**Fig. 1. Signalling from injured hepatocytes and cholangiocytes to non-parenchymal cells in the fibrotic niche.** Injured hepatocytes activate immune cells through DAMPs-TLR9, IL-1β, IL-18 and CXCL10 and directly stimulate myofibroblast differentiation and activation through TGFβ1/2, SHH and PDGFRBB signalling. Immune cell-mediated mechanisms of myofibroblast activation include TGFβ1/2- and amphiregulin-mediated pathways. Ligands are represented in blue and receptors in red.
levels of TGFβ1. However BMDMs, which accumulate in chronic inflammation remain transcriptomically different from KCs, in a more pro-inflammatory state, as evidenced from mouse models. These monocyte-derived/bone marrow-dependent macrophages in NASH livers share striking similarities with macrophages in related extrahepatic compartments such as the bone marrow or adipose tissue, including a subset of “lipid-associated macrophages” with a unique metabolically activated phenotype.

The advent of single-cell RNA-sequencing (scRNAseq) has resulted in a profound change in our ability to understand the myeloid cell population within the human hepatic fibrotic niche at unprecedented resolution. Ramachandran et al. used scRNAseq technology to identify a disease-associated TREM2+/CD9+ macrophage population that was significantly expanded in human cirrhotic livers. Immunofluorescence staining demonstrated that these TREM2+/CD9+ macrophages were highly enriched in the fibrotic niche. Analysis of the transcriptome of these scar-associated macrophages demonstrated a phenotype analogous to TREM2+/CD9+ BMDMs in mice. In silico trajectory analysis, based on spliced vs. unspliced mRNA ratios, suggested that these cells were derived from blood monocytes. Gene ontology of the top differentially expressed genes identified pro-fibrogenic factors, such as IL-1β, TREM2 (triggering receptor expressed on myeloid cells 2), CCR4, C-C motif chemokine receptor (CCR)2, CXCL8, TNFSF12 and vascular endothelial growth factor (VEGF)A, which regulate scar-producing myofibroblasts, upregulate inflammation and promote angiogenesis. This high-resolution transcriptomic data also enabled in-depth ligand-receptor analyses, which identify differentially expressed ligands from cells within the fibrotic niche and pair them with cognate receptors expressed in cell populations within the dataset. This can be used to establish a ‘multi-lineage interaction’. Ligand-receptor pairs were localised to the fibrotic niche between scar-associated macrophages expressing TNFSF12 and PDGFβ with myofibroblast receptors TNFSF12A and PDGFRα, respectively. Cognate, pro-fibrotic myofibroblast receptors were also identified for secreted phosphoprotein 1 and IL1β. Using scRNAseq, Glass et al. measured significant changes in the gene expression of KCs in a NASH diet mouse model, including partial loss of KC identity, expression of TREM2 and CD9 and apoptosis. This may also have more direct consequences for metabolic injury in hepatocytes, since KCs, or at least their CD206+ESAM+ subset, actively participate in lipid metabolism. Identifying such cell-cell interactions within the fibrotic niche has clear translational potential for drug development. An emerging application of scRNAseq technology is ‘multi-omics’, combining methods such as scRNAseq and spatial transcriptomics, whereby the single-cell transcriptomes are mapped on a tissue section. Using these methods, Guilliams et al. localised unique populations of KCs and bile duct macrophages. They identified key interactions between KCs and HSCs via the ALK1-BMP9 and 10 axis and showed that the bile duct macrophages could be activated by exposure to lipids.

Dendritic cells and their interactions

Dendritic cells (DC) closely interact with T cells in their role as antigen-presenting cells and are subdivided into conventional DCs (cDC1 and cDC2) and plasmacytoid DCs (pDCs). Overall, DCs accumulate during NASH, but the data on specific sub-populations and their function remains controversial. Hepatic DC1 numbers have been suggested to increase or decrease in NASH. On the other hand, a NASH-promoting role of cDC1s was shown in mice by blocking X-C motif chemokine ligand 1 (XCL1), a chemokine with a key role in cDC1 recruitment, or by genetic depletion of cDC1 expressing XCR1, the receptor for XCL1. Thus, the XCL1-XCR1 axis seems to be a key mediator of hepatic cDC1 recruitment in NASH.

Innate lymphoid cells and their interactions

Innate lymphoid cells (ILCs) are a population of tissue-resident immune cells that lack T- and B-cell receptors, which also include natural killer (NK) cells in addition to other functionally diverse ILC subtypes. NK cells are an innate immune cell type that demonstrate cytolytic activity towards stressed or apoptotic cells. They are integral modulators of the inflammatory micro-environment and play an important role in NASH. NK cells are activated by inflammatory cytokines including IL-12, IL-15 and IL-18 and subsequently upregulate inflammation via interferon-γ. Importantly, NK cells inhibit and indeed kill activated mesenchymal cells and myofibroblasts. However, myofibroblasts that fail to enter senescence and continue to produce scar tissue are resistant to NK activity and perpetuate fibrosis even after the inflammatory stimulus has reduced. Moreover, NK cells, via crosstalk with macrophages, attenuate NAFLD-induced fibrosis by regulating M1/M2 polarisation. IL-33-mediated expansion of ILC2s was shown to promote toxin-induced liver fibrosis through an IL-13-IL4R-STAT6 pathway. Similarly, IL-33 treatment aggravated hepatic fibrosis in NASH but at the same time attenuated hepatic steatosis and serum alanine aminotransferase. However, deficiency of IL-33 did not affect NASH-induced fibrosis. Together, these data suggest complex roles for IL-33 and ILC2 in NASH and possibly thresholds for IL-33 that need to be surpassed to modulate fibrosis. ILC3 significantly increase in fatty liver, and RORγt Ki/Ki mice, which are deficient in ILC3, showed increased hepatic steatosis and fibrosis. Accordingly, IL-22, which is produced by ILC3 and signals through the IL-22/IL-22R1/IL-10R2 complex, ameliorated experimental liver fibrosis by inducing HSC senescence via upregulation of STAT3.

Unconventional T cells, which mostly recognise lipids, small molecule metabolites and modified peptides in an MHC-independent manner, include natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells and γδ T cells. In the liver, unconventional T cells represent the majority of T cells. With most functional studies done in mice, it is important to recognise that NKT cells are abundant in livers of mice but rare in humans, and vice versa, MAIT cells are abundant in human but rare in mouse livers. Hepatic NKT cell numbers increase in human and mouse NASH, and NASH and fibrosis are reduced in CD1d-deficient mice, which lack NKT cells. Mechanistically, several ligand-receptor pairs mediate the effects of NKT cells, including LIGHT-HVEM (which promotes hepatocyte steatosis) and CXCL16-CXCR6 (which promotes hepatic NKT cell recruitment and liver fibrosis). The role of MAIT cells in NASH remains poorly understood. While studies in MR1-deficient mice (which lack MAIT cells) and Vα19TCRtg mice (which have increased MAIT cells), suggest a pro-inflammatory and pro-fibrogenic role of MAIT cells in toxic liver fibrosis (mediated by interactions with HSCs via TNF and its receptors, as shown in co-cultures), studies in an methionine-choline deficient diet NASH...
intercellular adhesion molecules and vascular cell adhesion molecules. Neutrophils are also important components of the inflammatory response in NASH. Macrophages mediate the adhesion, migration, and activation of neutrophils via CD44, and macrophage antigen 1, L-selectin, E-selectin, P-selectin, and the integrin superfamily. In turn, neutrophils activate KCs and endothelial cells, resulting in the upregulation of intercellular adhesion molecules and vascular cell adhesion molecules. Myeloperoxidases secreted by neutrophils are associated with steatosis and inflammation. Neutrophils are also the main producer of IL-17A, a cytokine that promotes Th17 production and upregulates the expression of TGFβ receptors on myofibroblasts. In a positive amplification loop, TGFβ in turn induces the expression of IL-17A, when expressed in the presence of other pro-inflammatory cytokines. These include caspase 1, NOD receptors, LRR (leucine-rich repeats) receptors, the NLRP3 pathway and NF-κB, which have all been identified as upstream activators of the IL-17A–TGFβ axis.

Platelets and their interactions

In NASH, platelets interact with liver-resident KCs to promote the recruitment of CD8 T cells and NKT cells, depending on interactions between platelet-expressed GPⅢa and von Willebrand factor, thereby promoting disease. Moreover, platelets are a rich source of growth factors, including PDGF, and thereby promote proliferation of PDGFR-expressing cells such as HSCs in non-NASH settings.

Neutrophils and their interactions

Neutrophils are also important components of the inflammatory cell response in NASH, which is characterised by the early infiltration of neutrophils. The number of these cells, as well as the neutrophil-to-lymphocyte ratio correlates with serum alanine aminotransferase levels and significantly predicts fibrosis in humans. There is significant crosstalk between macrophages and the neutrophils that they recruit during NASH. Macrophages mediate the adhesion, migration and activation of neutrophils via CD44, and macrophage antigen 1, L-selectin, E-selectin, P-selectin, and the integrin superfamily. In turn, neutrophils activate KCs and endothelial cells, resulting in the upregulation of intercellular adhesion molecules and vascular cell adhesion molecules.
and also secrete IL-17 that exacerbates steatosis and inflammation. The role of B lymphocytes in NASH is also emerging, with respect to both the production of circulatory IgG against oxidative stress-derived epitopes as well as potential roles within the fibrotic liver parenchyma itself. B cells exhibiting a pro-inflammatory phenotype (IL-6 and TNF-α expressing) accumulate in the liver lobules of rodent NASH and NASH gut dysbiosis models. The immunological microenvironment of the hepatic fibrotic niche is summarised in Fig. 2.

Mesenchymal cells and their interactions

The major classes of mesenchymal cells in the healthy liver include HSCs, portal fibroblasts (PFs) and vascular smooth muscle cells. Following liver injury, hepatic myofibroblasts are the primary scar-producing cells. Myofibroblasts express high amounts of fibrillar collagens, ECM proteins, inflammatory cytokines and chemokines. Myofibroblasts have been shown to differentiate from HSCs or PFs dependent on the model of liver injury. In rodent models of parenchymal injury, it is predominantly HSCs that activate and differentiate into myofibroblasts. HSCs are resident perisinusoidal mesenchymal cells that radiate cytoplasmic projections throughout the space of Disse. The perisinusoidal fibrosis in early human NASH is thought to be produced by activated HSCs/myofibroblasts. Upon injury, HSCs become activated, increasing their contractility, secreting inflammatory mediators, and synthesising ECM components. In acute injury, wound healing is dynamic and reversible. Deposition of the ECM is crucial for mechanically stabilising injured tissue, enabling effective migration of immune, mesenchymal and endothelial cells into the repairing tissue. However, when injury is repetitive, ECM components continue to accumulate and fibrosis occurs. Hepatocytes, macrophages, cholangiocytes, endothelial and immune cells can all promote or inhibit the activation of HSCs via multiple mechanisms (see Fig. 3). In response to growth factors such as PDGF from macrophages, connective tissue growth factor (CTGF) from mesenchyme/endothelial cells, VEGF and TGFβ1, quiescent HSCs differentiate into myofibroblasts. TGFβ1 binding and phosphorylation of downstream SMAD proteins promotes the secretion of the fibrillar collagens, type 1 and 3 into the ECM, replacing the basement membrane collagens. TGFβ1 is primarily secreted by epithelial cells and KCs. As the ECM is established and stiffens, mechanical strain provides a direct mechanism for the conversion of latent TGFβ1 into its active form. Myofibroblasts continuously regulate matrix deposition and turnover through the actions of MMPs and tissue inhibitors of metalloproteinases (TIMPs). Activated HSCs and myofibroblasts produce multiple chemokines, including CCL2, CCL3, CCL5, CXCL1, CXCL8, CXCL9 and...
CXCL10 that orchestrate inflammation through leukocyte recruitment. HSCs also secrete macrophage colony stimulating factor (CSF), IL-6, RANTES, CCR2 and CCR5, amplifying the acute response to inflammation. Macrophages then secrete IL-1β which may promote the survival of HSCs and increase the pool of collagen-producing cells.

HSC-mediated, fibrogenic signalling pathways include TGFA, keratinocyte growth factor, thrombin, fibroblast growth factor (FGF) and epidermal growth factor which cause proliferation of hepatocytes, cholangiocytes and mesenchymal cells. Similar to macrophages, HSCs also express TLR4 and therefore play a role in TGFβ1 signalling in response to the increased levels of DAMPs in NASH. HSCs modulate the ECM and the formation of scar tissue via 2 types of collagen receptors, integrins and discoidin domain receptors (DDRs). Both receptors are independently involved in regulation of cell adhesion, differentiation, proliferation of myofibroblasts and migration of both myofibroblasts and macrophages. HSCs are also mediators of hepatic regeneration, as the main source of hepatocyte growth factor.

Other signalling pathways that directly or indirectly activate HSCs include: TAZ, which is specifically expressed in NASH hepatocytes; Notch, which influences HSC cell fate toward myofibroblasts; osteopontin, which is induced by Notch and TAZ signalling and hedgehog pathways including Indian hedgehog and sonic hedgehog, which are expressed in ballooned NASH hepatocytes and closely correlate with lymphocytic infiltration, ductular reaction and ECM collagen deposition.

scRNAseq allows for high-resolution transcriptomic profiling of mesenchymal subpopulations during liver fibrosis, and subsequent ligand-receptor analysis of the non-mesenchymal populations interacting with these mesenchymal subpopulations. For instance, scRNAseq data from mouse fibrosis models revealed that chemokine/cytokine release and ECM production appears to be diversified between HSC/myofibroblast subpopulations. Ramachandran et al. used this approach to identify a distinct population of scar-associated mesenchymal cells in the human liver expressing high levels of fibrogenic genes and the myofibroblast marker, PDGFRα. These myofibroblasts were topographically restricted to fibrotic septae and ligand-receptor analysis revealed a number of pro-fibrogenic pathways including TNFRSF12A, PDGFR and Notch. These receptors were paired to their corresponding ligands in scar-associated macrophages and scar-associated endothelia.

scRNAseq has also been used to categorise murine HSCs into central vein and portal vein-associated functional zones, with central vein-associated HSCs as the primary fibrillar collagen producers in a mouse model of centrilobular fibrosis. A further scRNAseq-based study of mouse hepatic mesenchyme also demonstrated zonation of HSCs across the healthy liver lobule, and following induction of NASH, the authors observed 4 distinct HSC clusters, including 1 representing the classic fibrogenic myofibroblast. The 3 other HSC clusters comprised a proliferating cluster, an intermediate activated cluster, and an immune and inflammatory cluster. Interestingly, livers with NASH regression had 1 cluster of activated HSCs, which was similar to, but distinct from, the quiescent HSCs. MacParland et al. found that quiescent and activated HSCs were transcriptomically distinct, with activated HSCs downregulating vitamin A storage-related genes, such as LRAT (lecithin retinol acyltransferase) and RBP1 (retinol binding protein 1), and upregulating pro-fibrogenic genes, such as collagen type I x1, TIMP, CTGF, TGFβ1 and tenasin C. They also identified a subgroup that may represent senescent HSCs, having completed their activation phase, expressing IL-32, CSF1, TNFSF10, CCL2, IL-6ST. These subpopulations were identified in healthy human livers, facilitating exploration of the role of HSCs in liver homeostasis. The transcriptomes of NASH-associated mesenchyme subpopulations identified using scRNAseq can be analysed in-depth for ligand-receptor interactions with other cell lineages. Wang et al. identified a number of potential fibrosis-associated myofibroblast genes, including AEBP1 (AE binding protein 1), LARP6 (1a ribonucleoprotein 6, translational regulator) and PRRX1 (paired related homeobox 1), as well as pro-fibrotic signalling axes, such as ITGAV-LAMC1 and NOTCH2-DLL4. As well as collagen production, ligand-receptor analysis indicates that activated HSCs play a complex role in fibrotic signalling through the ECM secretome itself: secreted proteins such as collagen serving both a structural and a signalling function with cognate receptors such as ITGAV1 integrin subunit x1 or DDR2. They also have activating receptors for a number of vasoactive proteins such as VIPR1 (vasoactive intestinal protein receptor 1) and AGTR1A (angiotensin II receptor type 1a).

PFs represent an additional mesenchymal population in the liver, however their role in NASH is much less well studied than that of HSCs. PFs are a key myofibroblast precursor in cholestatic liver injury, and populations of PFs have been shown to expand in murine models of biliary injury, expressing high levels of fibrillar collagen at single-cell resolution. These cells are distinct from HSCs with no vitamin A storage and express unique markers such as THY1, fibrulin (FBN1), FBLN2, MFAP4 (microfibril associated protein 4) and GAS6 (growth arrest specific 6).

### Endothelial cells and their interactions

There are several endothelial cell subpopulations that line the hepatic vasculature and lymphatic vessels. Liver sinusoidal endothelial cells (LSECs) are localised along the sinusoids and form an interface between blood arriving from the gut and the hepatic parenchyma. In health, LSECs regulate anti-inflammatory and antifibrotic signalling by preventing KC and HSC activation via nitric oxide, cGMP and VASP (vasodilator stimulated phosphoprotein). Like KCs, LSECs express TLR4 receptors that recognise bacterial lipopolysaccharides and also express unique scavenger receptors such as CLEC4G that mediate endocytosis. As well as endocytosis and antigen presentation, LSECs mediate adhesion and migration of leukocytes into the parenchyma via chemokines such as vascular adhesion protein 1 (monocyte and T-cell recruitment), CCL2 (monocyte recruitment), CXCL10 (T-cell migration) and CXCL16 (NK(T) cell migration). Therefore, LSECs play a key role in immunomodulation in the liver and many of these leukocyte migration axes are upregulated in NASH.

One key observation in the progression of cirrhosis is the loss of LSEC fenestration, termed ‘capillarisation’. Not only does this contribute to portal hypertension and reduce the efficiency of the liver to interface with portal blood, but it also prevents the export of lipids such as VLDL back into the circulation. LSEC capillarisation is therefore closely linked to steatosis in NASH. Possible triggers for LSEC capillarisation include gut microbiota and dietary macronutrients. Importantly, capillarisation is associated with a switch from fibrosis-restricting to fibrosis-permissive. Moreover, LSECs control the balance between liver regeneration and fibrogenesis via a pro-regenerative CXCR7-ld1 pathway and a pro-fibrotic FGFR1-CXCR4 pathway. Scar-associated LSECs have been shown by scRNAseq to expand in...
human cirrhosis. These cells have a PLVAP+ and ACKR1+ transcriptome and demonstrate a number of pro-inflammatory ligand-receptor interactions, such as TGBF1-TGBFR3, JAG1-NOTCH1 and CCL2-ACKR1.75

ScRNAseq has also revealed that, like hepatocytes, LSECs appear to be highly zonated in homeostasis and lose this zonation in NASH cirrhosis. Portal-central zonation is in part mediated by the Wnt-β-catenin pathway and influenced by the intestinal microbiome. LSECs in pericentral regions have been shown to be more vulnerable to damage associated with cirrhosis.144–146 This may partly explain why restoration of a gut microbiota associated with health has been shown to reverse portal hypertension in rat models of NASH.147

Long distance interactions in NASH: Gut dysbiosis, adipose tissue and bile acids

In NASH, an interplay of genetic, metabolic and microbiological factors combine to progress liver pathology from steatosis to fibrosis in what is seen as a ‘multiple hit’ model. ‘Dysbiosis’, defined as an altered disease-associated microbiome, has been shown to increase with the severity of NASH in both rodents and in humans. This is paralleled and closely related to increased intestinal permeability, bacterial translocation into the portal system and activation of pro-inflammatory pathways.148 In chronic liver disease, bacteria and microbial products, collectively known as microbiota-associated molecular patterns, translocate across the intestinal barrier owing to intestinal barrier disruption. Microbiota-associated molecular patterns such as bacterial lipopolysaccharide accumulate in the portal vein and have been shown to act as ligands for TLR2 and TLR4. These activate pro-inflammatory pathways leading to secretion of cytokines and chemokines from various cell types in the liver, including endothelia, myofibroblasts and hepatocytes.125,149,150 All known human TLRs are expressed by HSCs, indicating the importance of their interaction with the gut microbiome.160,161 In mouse models, the severity of experimental liver fibrosis can be attenuated either by blocking TLR4 signalling or reducing microbial load with antibiotics. TLR4 also downregulates a TGFβ1 decoy receptor, thereby sensitising HSCs to TGFβ1.152 TLR4 signalling includes a signalling adaptor called TRIF (alternatively called TICAM1). TRIF deletion in a model of diet-induced NASH increased hepatic fibrosis but reduced hepatic steatosis.153 Gut dysbiosis also leads to changes in bacterial choline metabolism and subsequent dysregulation of farnesoid X receptor (FXR) and FGF19 that leads to myofibroblast activation.154 Moreover, the gut microbiota is linked to NASH via immune cells with key functions in the regulation of NASH and fibrosis, such as γδT and MAIT cells, as discussed above.69

The metabolic syndrome also alters and upregulates fibrotic cell-signalling in the liver. For example, fatty acids amplify the inflammatory response by interacting with transcription factors such as HNF4α and TLRs.155,156 The adipokine, Leptin, which is produced by adipocytes and increased in obesity, amplifies profibrotic signalling during liver injury in several ways, including increased production of TGF-β1 from KCs,157 and direct effects on HSCs.158 Targeting the sequealae of the metabolic syndrome is a potential therapeutic strategy with peroxisome proliferators-activated (PPAR) receptors inducing fatty acid oxidation and reducing metabolic strain. The PPARγ nuclear receptor also downregulates collagen expression as well as HSC activation.159

A further feature of progressive liver fibrosis is the disruption of bile acid (BA) homeostasis. Alongside their role in absorption of lipids and lipid-soluble vitamins, BAs act as ligands. BAs suppress their own synthesis by binding to BA receptors, most prominently the FXR, which is critical for regulating their enterohepatic circulation. BA receptors have multiple downstream functions, including lipid regulation, glucose metabolism, modulation of inflammation and fibrosis. FXR is expressed in the intestine, where it contributes to the gut-liver axis through release of FGF19. In addition, scRNAseq has revealed that high expression of FXR contributes to HSC activation and collagen production in cholestatic liver disease.160 Another important BA receptor is TGR5 (also known as GPBAR1), a surface receptor that has been linked with steatosis and inflammation, which has a direct role in NLRP3 macrophage polarisation in NASH.161 Furthermore dysregulated BA homeostasis alters the immune cell composition. In a recent study, patients with NAFLD were found to have taxonomic alterations in the intestinal microbiome favouring short chain fatty acid-producing bacteria, higher faecal levels of choline metabolites and reduced numbers of regulatory T cells.162 The FXR agonist obeticholic acid has demonstrated clinical efficacy in NASH, with improvements in liver steatosis, liver inflammation and fibrosis on sequential biopsies.163

Conclusions and future strategies

Despite advances in our understanding of the pathophysiology of NASH, there are still no FDA- or EMA-approved therapies for NASH or NASH-induced liver fibrosis. There are currently only 2 licensed antifibrotic medications (nintedanib and pirfenidone) in the fibrosis space, both developed to treat idiopathic pulmonary fibrosis.164 However, recent step changes in scientific approaches and methodologies including the rapidly evolving field of single-cell genomics (Table 1), have enabled investigation of the cell states and subpopulations which inhabit the human hepatic fibrotic niche at unprecedented resolution. It is hoped that these approaches will help drive a new era of precision medicine in the search for effective therapies for NASH, and many other forms of liver disease. Furthermore, multimodal single-cell approaches are swiftly gaining traction within hepatology, allowing ‘combination readouts’ from the same cell, for example simultaneous acquisition of transcriptomic and epigenomic information, again maximising our ability to identify the key therapeutic targets in pathological cells.165,166 In addition, linkage of genome-wide transcriptomic information with spatial context is now possible, with the advent of spatial transcriptomics, allowing ‘on-slide’ RNA-sequencing of tissue sections at ever-increasing resolution as the technologies in this area advance and mature.167

Again, deploying these types of technologies should help drive forward precision medicine in hepatology. Together, these approaches may provide a vast array of novel candidate targets. For example, ligand-receptor analysis (using curated databases of ligand-receptor interactions) can simultaneously compare genes expressed within different cellular subpopulations present within the NASH fibrotic niche. This interactome modelling of ligand-receptor pairs between pathogenic subpopulations in NASH and other human liver diseases could therefore provide a molecular framework for the therapeutic targeting of key pathogenic cell populations.168,169 Interrupting disease-promoting cell-cell interactions via specific ligand-receptor pairs may improve key aspects of NASH such as fibrogenesis and...
inflammation. Likewise, understanding key regulatory pathways within specific disease-promoting cell populations or subpopulations may enable the pharmacological or genetic targeting of these populations. High-throughput methods which enable investigation of the function of specific genes within an interactome are gaining prominence. Perturb-seq facilitates the analysis of thousands of CRISPR-mediated gene perturbations at single-cell resolution and can also be applied to common and rare cell populations. These latter technologies also have enormous potential for precision drug design. Analysis of cell trajectories, in combination with above analyses on ligand-receptor interactions and regulatory pathways, may pave the way for treatments that interrupt differentiation of specific cell types into disease-promoting states, or, vice versa, stimulate trajectories towards a regression/resolution-promoting state. Better understanding of epigenetic regulation of disease-promoting mechanisms may enable the development of therapies that target the epigenome in NASH. Furthermore, bioinformatic methodologies are continually being refined to keep pace with the rapid advances in multimodal dataset acquisition.

Global, highly collaborative and publicly accessible multimodal databases, such as the Human Cell Atlas, are also playing a very important role in disseminating data, and importantly, making it easily accessible and usable to researchers around the world. It is now commonplace for research groups to release their single-cell datasets in easy-to-use web browser formats, thereby maximising utilisation of these expansive datasets within the scientific community. Likewise, a wide range of constantly updated analysis tools for ligand-receptor interactions, gene regulatory network analysis, cell trajectories and drug mechanism of action, amongst many others, will undoubtedly improve our understanding of NASH in the coming years. Multimomic approaches that integrate transcriptomic, epigenetic and spatial analyses will allow us to precisely define the constituents of the cellular interactome of NASH and increase our understanding of how these various subpopulations within the fibrotic niche communicate and interact to drive and also resolve fibrosis. The convergence of these novel approaches represents an extraordinary opportunity to perform functional in silico analyses of the cellular and molecular mechanisms that regulate human liver disease at unprecedented resolution. In sum, these developments should pave the way towards the rational design of effective new therapies for patients with NASH, targeting specific cell–cell communication pathways, possibly with combination therapies tailored to specific stages of liver disease.

Abbreviations

BAs, bile acids; CCL, C-C motif chemokine ligand; CCR, C-C motif chemokine receptor; CLD, chronic liver disease; CTGF, connective tissue growth factor; CXCL, C-X-C motif chemokine ligand; CXCR, C-X-C motif chemokine receptor; DAMP, damage-associated molecular pattern; ECM, extracellular matrix; ER, endoplasmic reticulum; FGF, fibroblast growth factor; FXR, farnesoid X receptor; HSCs, hepatic stellate cells; IL, interleukin; ILC, innate lymphoid cell; KCs, Kupffer cells; LSECs, liver sinusoidal endothelial cells; MAF, mucosal-associated invariant T; MAMPS, microbiota-associated molecular patterns; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NK(T), natural killer (T); NLR, Nod like receptors; PDGF, platelet-derived growth factor; PFs, portal fibroblasts; SASP, senescence-associated secretory phenotype; TGF, transforming growth factor; TLR, Toll-like receptor; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

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Table 1. Summary of some of the current single-cell genomics technologies.

| Technology | Examples | Application | Advantages/Disadvantages |
|------------|----------|-------------|--------------------------|
| Single-cell RNA-sequencing | 10X Chromium<sup>®</sup> | Allows rapid and high-throughput single-cell RNA-sequencing | Single-cell transcriptome resolution at scale Potential for cell-type and cell-stress bias from live cell isolation during tissue digest |
| Single-nuclei RNA-sequencing | 10X Chromium<sup>®</sup> | Allows rapid and high-throughput single nuclei RNA-sequencing | Can use frozen tissue, excellent utility in single hepatocyte transcriptome generation Potentially lower read depth (although recent head-to-heads with single-cell RNAseq are comparable)<sup>71</sup> |
| Multi-ome transcriptome-epigenome sequencing | Single-cell ATAC-seq | Combined epigenetic and transcriptomic profiling from the same cell | Can also be combined with single-cell RNA-sequencing databases to analyse epigenetic regulation of genes and identify regulators such as transcription factors. ATAC-seq data can potentially be very sparse. |
| Spatial transcriptomics | 10X Visium<sup>®</sup> MERFISH | Maps gene expression (genome-wide – Visium, or high plex – MERFISH) spatially within FFPE or frozen tissue sections | Allows interrogation of in situ gene expression at very high plex or genome-wide. A very rapidly evolving field with current pros and cons to most approaches – best approach depends on the biological question. |
| Ligand-receptor interactome analysis | CellPhoneDB Nichenet | Identifies ligand-receptor or regulatory gene interactions within a single-cell database | Can be applied to large transcriptomic databases to identify significant ligand-receptor interactions between different cell subpopulations. Will likely be very useful algorithms in the context of spatial transcriptomic databases as well. Potentive ligand-receptor interactions require wetlab validation/interrogation. |
| High-throughput CRISPR perturbed phenotype analysis | Perturb-seq | Analysis at scale of CRISPR-mediated gene perturbations at single-cell resolution | High-throughput functional analysis of the transcriptome with potential to underpin precision drug design. |

ATAC-seq, assay for transposase-accessible chromatin using sequencing; FFPE, formalin fixed paraffin embedded; MERFISH, multiplexed error-robust fluorescence in situ hybridisation.
Conflict of interest

The authors declare no conflicts of interest that pertain to this work.
Please refer to the accompanying ICMJE disclosure forms for further details.

Authors’ contributions
S.J.W., F.T., R.F.S. and N.C.H. contributed equally to the writing and editing of all aspects of this review.

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Author names in bold designate shared co-authorship

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