DR. SCOTT LAURENCE FRIEDMAN (Orcid ID : 0000-0003-1178-6195)

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Hepatic Fibrosis 2022:

Unmet Needs and a Blueprint for the Future

Scott L. Friedman[1] and Massimo Pinzani[2]

1. Division of Liver Diseases, Icahn School of Medicine at Mount Sinai, New York, NY USA
2. University College London, Institute for Liver and Digestive Health, London, UK

Correspondence address:

Scott L. Friedman MD

Box 1123, Icahn School of Medicine at Mount Sinai

1425 Madison Ave, Room 1170C

New York, NY 10025, USA

Email: Scott.Friedman@mssm.edu

Massimo Pinzani, MD, PhD

UCL Institute for Liver and Digestive Health

Royal Free Hospital

London NW32PF, United Kingdom

Email: m.pinzani@ucl.ac.uk

Abstract

Steady progress over 4 decades towards understanding the pathogenesis and clinical consequences of hepatic fibrosis has led to the expectation of effective anti-fibrotic drugs, yet none has been approved. Thus, an assessment of the field is timely to clarify priorities and accelerate progress. Here we highlight the successes to date, but more importantly identify gaps and unmet needs, both experimentally and clinically. These include the need to better define cell-cell interactions and etiology-specific elements of fibrogenesis and their link to disease-specific drivers of portal hypertension. Success in treating viral hepatitis has revealed the remarkably capacity of the liver to degrade scar in reversing fibrosis, yet we know little of the mechanisms underlying this response. Thus, there is an exigent need to clarify the cellular and molecular mechanisms of fibrosis regression in order for therapeutics to mimic the liver's endogenous capacity. Better refined and more predictive in vitro and animal models will hasten drug development. From a clinical perspective, current diagnostics are improving but not always biologically plausible or sufficiently accurate to supplant biopsy. More urgently, digital pathology methods that leverage machine learning and artificial intelligence must be validated in order to capture more prognostic information from liver biopsies and better quantify the response to therapies. For more refined treatment of NASH, orthogonal approaches that integrate genetic, clinical and pathological datasets may yield treatments for specific sub-phenotypes of the disease. Collectively, these and other advances will strengthen and streamline clinical trials, and better link histologic responses to clinical outcomes.

1. Introduction

The field of fibrosis is ripe for success. After ~40 years of steady progress in basic, translational and clinical research, there is a rich appreciation of its pathogenesis and contribution to end-stage liver disease. Yet, success in treating fibrosis has been harder won than anyone

anticipated, and early optimism was premature as there are still no approved antifibrotic therapies for liver disease. At this juncture, it is timely to assess where we are in the path towards success, what we have learned and what are the current unmet needs – both clinical and investigational - that will finally translate into effective therapies. In this review, we seek to frame our understanding of hepatic fibrosis in the context of current concepts and unmet needs, highlighting areas that require further investigation in hopes of accelerating success in treating fibrosis that delay or prevent the complications of end-stage liver disease and improve outcomes.

Progressive fibrosis typically follows long-standing liver damage due to infectious (hepatitis B–HBV- and C–HCV-viruses), toxic/drug-induced (mainly alcohol-induced), metabolic (non-alcoholic fatty liver disease, or NAFLD), cholestatic or autoimmune insult. Eventually, fibrosis may lead to clinically evident cirrhosis and hepatic failure. Cirrhosis is defined as an advanced stage of fibrosis, characterized by the formation of regenerative nodules of liver parenchyma that are separated by, and encapsulated in, fibrotic septa (1).

Historically, hepatic fibrosis was long considered a passive and irreversible process resulting from the collapse of hepatic parenchyma and its gradual replacement with collagen-rich tissue (2), but countless studies have underscored the importance of active fibrogenesis that leads to accumulation of extracellular matrix (scar). Remarkably, the pathogenesis of hepatic fibrosis received little attention until the 1980s, when hepatic stellate cells (HSC), formerly known as Ito cells, lipocytes or fat-storing cells, were identified as the dominant cellular source of extracellular matrix (ECM), or scar (3). The development of reproducible methods to isolate these cells from rodents and humans with a high purity facilitated their investigation, initially in isolated HSCs following their 'activation' in culture, and subsequently through analysis *in vivo* (4).

Fibrogenesis, or the generation of scar, is a dynamic process in chronic injury characterized by continuous accumulation of fibrillar extracellular matrix (ECM) associated with concurrent matrix degradation and remodeling. Like fibrogenic disorders in other organs and tissues, fibrosis is a well-orchestrated wound-healing response. The response to chronic injury differs substantially from an acute tissue insult, in which there is no progressive scarring. From an evolutionary perspective,

fibrogenesis is a logical response to tissue damage by encapsulating injury and maintaining tissue integrity for a sufficient enough time to enable the propagation of the species. However over time, the fibrotic response impairs hepatic regeneration and shortens life expectancy, albeit in a time-frame that no longer jeopardizes reproduction. In clinical terms, moderate tissue fibrosis is typically not associated with significant clinical signs or decreased organ function but is nonetheless an important determinant of prognosis over decades, and sometimes in shorter intervals.

2. Basic principles

Activated HSCs are the key effectors of fibrogenesis through increased deposition of fibrillar ECM and by releasing cytokines, chemokines and other mediators establishing, together with inflammatory cells, a pro-fibrogenic environment that negatively affects the regeneration of the liver parenchyma (5-7).

Although HSCs are the main source of myofibroblasts in the liver (8, 9), other cell types contribute to the pool of fibrogenic myofibroblasts in chronic liver disease. In particular, portal myofibroblasts are located around bile ducts and generate biliary fibrosis (10). Bone marrow derived myofibroblasts have been implicated to a very minor extent in fibrosis as well (11).

Activation of HSC is stimulated by damaged and apoptotic hepatocytes through several converging pathways. These include: i) disruption of the normal ECM of the space of Disse as a consequence of hepatocyte damage and inflammatory infiltration (12, 13); ii) release of reactive oxygen species (ROS) and other fibrogenic/pro-inflammatory mediators (14, 15); iii) recruitment of immune cells, which in turn sustain HSC activation (6, 16).

Attention has focused on the pro-fibrotic microenvironment of the liver, with increasing interest in the role of immune cells and specific subsets of macrophages regulating the progression or the regression of fibrosis (see below)(17) and the role of intestinal microbiota (18). Other fibrogenic stimuli include tissue hypoxia with the establishment of an anaerobic pro-inflammatory

environment (19) and the influence of epigenetic modifications (20) in conditioning the progression of fibrosis.

Fibrogenesis during chronic injury increases the amount, composition and distribution of different ECM components. In the healthy liver, the ECM in the space of Disse, the space between endothelial cells and hepatocytes, mainly consists of collagen IV and laminin. During progressive fibrosis, fibrillar collagens, especially collagens I and III, replace these low-density basal membrane-like structures. Recent successes in decellularizing normal and fibrotic human liver tissue has yielded insights into the healthy and disease-specific hepatic "matrisome", (i.e. the biochemical and biomechanical properties of the ECM), and has clarified our understanding of how cells interact with, and respond to either a healthy or pathologic tissue microenvironment (21). In particular, the unique disease-specific ECM environment affects both hepatocyte differentiation and function and helps explain the processes that promote the progression to cirrhosis and the development of hepatocellular carcinoma (HCC) (22).

Liver sinusoidal cells (LSEC) have unique features including the presence of pores, or fenestra, which are typically lost as subendothelial ECM accumulates, in a process termed 'capillarization' (23). LSECs are implicated in maintenance of HSC quiescence, cellular cross-talk and support of liver regeneration (24-26). Although some studies have begun to define LSEC responses using single cell methods (27-28), few have characterized the phenotypic features of LSECs in normal liver function, and how they contribute to hepatic injury as well as to fibrosis progression and regression.

Major technical advances in capturing single-cell transcriptomes both in tissues and *in situ* are already yielding unprecedented clarity about the function of individual cells, their heterogeneity, and evolution during disease progression in both animal models and human liver [see Section 7, below and (29, 30)

Unmet needs

Based on the progress of the past 40 years, new opportunities can provide a deeper and more accurate understanding of fibrogenesis in human liver disease.

A. Despite the expansive knowledge about the cellular mechanisms of liver fibrosis, this knowledge is mainly hepatic stellate cell-centric. Transcriptomic analyses of individual cells can provide unprecedented insights into interactions between all the cell types that comprise fibrogenic response and evolving microenvironment in progressive disease. The "holy grail" is to leverage these new tools to better understand the intimate relationships between tissue damage, regeneration, fibrogenesis and cancer.
B. Many principles of fibrogenesis are drawn from two dimensional models in which HSC are activated and cultured on plastic, which may lead to erroneous conclusions about their behavior *in vivo*. Greater refinement and standardization of 3D *in vitro* models that faithfully recapitulate the microenvironment of human liver diseases will accelerate progress.
C. The cellular and molecular biology of liver fibrogenesis should distinguished between "core" mechanisms, (i.e. common to fibrogenic diseases affecting different organs and characterized by an evolutionary role), and "regulatory" mechanisms, which are more tissue and disease specific (31).
D. Tools are now available for in-depth molecular and functional characterization of both macrophage and LSEC subsets in liver injury, fibrosis and liver cancer. Efforts to link these cell types to specific tissue responses will likely uncover important new pathways and therapeutic targets.

3. Etiology-specific features of hepatic fibrosis and cirrhosis

There are distinct patterns of fibrosis leading to cirrhosis linked to the underlying etiology (4) (Figure 1). For example, biliary fibrosis, resulting from the dual proliferation of reactive bile ductules and periductular myofibroblast-like cells at the portal-parenchymal interface, leads to fibrosis developing in a portal-to-portal direction that generates portal-portal septa surrounding liver nodules, where the central vein and its connections with the portal tract are preserved until late stages. In contrast, in chronic viral hepatitis, the pattern of fibrosis (termed "post-necrotic") results

from portal-central (vein) bridging necrosis, thus creating portal-central septa characterized by evident neo-angiogenesis, which leads to a derangement of the connection between the portal system and the hepatic vein. This vascular disconnection underlies early portal hypertension observed in this type of fibrogenic evolution. The so-called central-to-central (vein) fibrogenic evolution is typically seen in venous outflow obstruction (e.g. chronic heart failure) and is characterized by the development of central-to-central septa and "reverse lobulation". Finally, pericellular and perisinusoidal fibrosis are typical of alcoholic and non-alcoholic fatty liver diseases, in which the deposition of fibrillar matrix is concentrated around the sinusoids (capillarization) and around groups of hepatocytes (chicken-wire pattern). These different patterns of fibrogenic evolution are related to: 1) the topographic localization of tissue damage; 2) the relative concentration of pro-fibrogenic factors, and; 3) the prevalent pro-fibrogenic mechanism(s). In addition, these different patterns may indicate the participation of different cellular effectors of fibrogenesis, or at least different subtypes of fibrogenic cells as suggested by a recent animal models using single cell RNA sequencing (32-34). The different zonal patterns of fibrosis and etiologies may also dictate the rate of progression of liver disease, the dynamics of the necro-inflammatory infiltrate and the onset and progression of portal hypertension.

The progression from hepatic fibrosis to cirrhosis is characterized by major structural changes including the capillarization of sinusoids, the formation of fibrous septa encircling regions of the liver parenchyma, and extensive neo-angiogenesis with the formation of intrahepatic vascular shunts between the portal and the hepatic vein systems. In addition, the extent of neo-angiogenesis is dependent on the different patterns of fibrogenic evolution, with the highest expression in the post-necrotic form following chronic HCV infection.

Altogether, these observations suggest that the type of cirrhosis based on etiology and pattern may influence clinical management and potential antifibrotic targets. Indeed, the term "cirrhosis" traditionally implies an adverse prognosis related to the complications of portal hypertension, liver cancer and organ failure. In broad clinical terms, cirrhosis is defined as "compensated" and "decompensated" based on the degree of portal pressure and the occurrence of clinical complications. However, this dichotomous classification is an oversimplification that

overlooks critical biological events including the presence and vigor of regeneration. In addition, it does not reflect the continuum of fibrosis progression, with a range of stage-specific therapeutic options (35). In this context, the definition of favorable or unfavorable endpoints, and the need for an integrated clinical-pathological assessment. Such an assessment should include etiology, grade of activity, co-morbidity, risk factors for malignancy and features potentially suggestive of progressive disease (36).

Regardless, the current stratification of cirrhosis, irrespective of the etiology, is still based on the detection and monitoring of portal hypertension. The gold standard for the assessment of portal hypertension is the Hepatic Venous Pressure Gradient (HVPG), i.e., the difference between the wedged (WHVP) and the free hepatic venous pressures. HVPG is the gradient between pressures in the portal vein and the intra-abdominal portion of inferior vena cava while WHVP actually reflects hepatic sinusoidal pressure and not the portal pressure itself. Whereas normal HVPG is 1 - 5 mm Hg, higher values connote the presence of portal hypertension, and HVPG > 10 mm Hg (termed 'clinically significant portal hypertension') is predictive of the development of complications of cirrhosis, including death. HVPG above 12 mm Hg represents the threshold level of portal hypertension that could lead to variceal hemorrhage.

Unmet needs

A. Based on the divergent patterns of fibrosis across etiologies, it is possible that the development of portal hypertension and its clinical features are also etiology-dependent. Since HVPG is the only direct standard measurement on which clinical decisions and non-invasive parameters are based, it may be necessary to "reset" HVPG thresholds according the etiology of cirrhosis. Indeed, recent data indicate that the classic HVPG thresholds, developed mostly in HCV cirrhosis, do not reflect the risk of clinical manifestations in NASH cirrhosis in which severe complications may develop when HVPG is still below 10 mm Hg (37).
B. The analysis of the ECM composition (matrisome) in cirrhosis may be different across etiologies and thus, clarification of etiology-specific protein signatures should be pursued (22). Using this approach, it may become possible to identify specific ECM

shedding fragments in plasma or urine to be employed as staging and/or prognostic disease-specific biomarkers.

4. Reversibility of Hepatic Fibrosis and Cirrhosis

The severity of hepatic fibrosis is among the strongest predictors of clinical outcomes in chronic liver diseases, especially NASH (38), and therefore current research is focused on determining when fibrosis improvement is still possible, and what are its underlying mechanisms. Compared to chronic injuries in other organs, fibrosis progression in liver is generally slow, evolving over decades. This slower progression likely reflects the remarkable regenerative capacity of the liver, and may also account for the dramatic improvement in fibrosis seen when the underlying cause is removed. Nonetheless, underlying mechanisms linking fibrosis and regeneration are scant. From a practical perspective, documenting the regression of fibrosis - defined as a reduction in extracellular matrix content - is most critical in patients with cirrhosis, with the expectation that clinical outcomes may improve. In a combined analysis of patients with NASH cirrhosis from two negative clinical trials, those whose cirrhosis regressed had significantly fewer clinical events (39). For patients without cirrhosis, a reasonable endpoint is simply attenuation of further progression, such that cirrhosis never develops. Indeed, prevention of progression to cirrhosis is viewed as a 'hard' clinical endpoint by regulatory agencies, since the diagnosis of cirrhosis confers a rising risk of complications (40).

Fibrosis regression in patients with chronic liver disease has been recognized for decades in patients where the underlying disease is attenuated, for example in those cured of HCV, following antiviral HBV suppression or after surgical biliary decompression in secondary biliary fibrosis, among others [reviewed in (41) and (42)]. Based on these clinical observations, combined with advances in the 1980's and '90s in isolating and characterizing individual cell types in liver, there were a flurry of studies at that time that sought potential mechanisms to explain these remarkable clinical observations. Indeed, a growing list of candidate proteases that degrade scar constituents and their cellular sources were described, leading to models of matrix degradation in liver in which both hepatic stellate cells and liver macrophages were implicated as sources of matrix

metalloproteinases. Activity of these enzymes is further regulated by relative concentrations of inhibitory molecules known as 'tissue inhibitors of metalloproteinases' or TIMPs. In liver injury, elevated production of TIMPs by stellate cells has been implicated as an important functional rheostat that constrains the activity of these MMPs (43, 44). A key study more recently established macrophages as a source of matrix proteases, by demonstrating that their depletion in mouse models affected the level of fibrosis regression (45). Additional work further defined specific subsets of macrophages critical for matrix degradation in mouse models, especially Ly6C^{lo} macrophages (46) and others [reviewed in (47, 48)]. Altogether, there is evidence for protease production by multiple cell types in the liver, including macrophages, neutrophils, dendritic cells, sinusoidal endothelium, biliary epithelium and progenitor cells (47, 49). Tantalizing evidence in early mouse models has reinforced that therapeutic promise of understanding protease regulation in liver based on evidence that either augmentation or attenuation of matrix protease activity could further influence net fibrosis accumulation (50, 51).

Despite this promise, a coherent understanding of which proteases, cells and inhibitors regulate fibrosis regression in human liver and experimental models remains elusive, and the field has largely moved on to other challenges in understanding liver biology. This sea change may have reflected the growing availability of knockout mouse models to study other, more tractable pathways, combined with failures of clinical trials using MMP inhibitors to attenuate other diseases like arthritis, which suggested that the biology of matrix degradation was more complex than originally thought (52). Indeed, the initial clinical studies of protease inhibitors or activators in other tissues – none of which succeeded - were probably quite naïve by assuming that there was sufficient knowledge about both the pleiotropic activities of MMPs and their inhibitors, and the native substrates of these enzymes *in vivo* (53).

Fast forward to 2021, and now the depth of knowledge about matrix degradation biology has expanded tremendously, which demands a re-engagement of liver investigators. We now know that MMPs are part of a large metzincin superfamily that is comprised of four subfamilies, matrixins, astracins, bacterial serralysins and adamalysins [see (48, 54) for excellent reviews]. Among human MMPs, there are six distinct groups including collagenases, stromelysins, gelatinases, matrilysins,

membrane bound metalloproteinases and others (48, 54). Moreover, the functions of these enzymes have expanded well beyond matrix degradation to include cleavage of cell surface molecules, activation of latent growth factors, and regulation of cell signaling, in addition to serving as markers of disease activity and prognosis (48, 55, 56). Among many examples, MMP-7 was uncovered in an unbiased proteomic screen as a prognostic marker of biliary atresia, hinting at novel biology of this molecule whose further elucidation could yield mechanistic insight into the disease (57).

There is also an emerging biology of specialized pro-resolving mediators (SPMs) that are implicated in resolution of hepatic inflammation, injury and fibrosis, especially in NASH (58, 59). These are a family of lipid molecules derived from omega-3 poly unsaturated acids with well-characterized receptors, which can significantly affect inflammatory cell infiltration and progression of liver disease. Just as there are 'lipotoxic species' whose evanescence makes them hard to detect in NASH, there are also salutary lipid species whose further characterization are fertile areas to explore as potential therapies. For example, a recent study identified one SPM, maresin-1, as preventing inflammation and NASH progression through its agonism of the nuclear receptor RORα in a murine model (60). It is unclear if SPMs contribute equally to antagonizing liver disease from other causes besides NASH, or if they directly impact activated stellate cells/myofibroblasts to reduce fibrogenesis or inactivate these cells; thus these are exciting questions to pursue.

In parallel to these advances, the development and application of single cell analytic technologies for liver have exploded in the past 2 years. A rapidly expanding number of datasets that reveal the complete cell-specific transcriptomes and proteomes of human liver disease and rodent models have created new opportunities to characterize ligand-receptor interactions and define therapeutic targets, among other applications (28-30, 61-64). Combined with techniques that include spatial transcriptomics and spatial proteomics, these technologies promise to revolutionize our understanding of disease mechanisms and drug development (65-68).

Unmet needs

Together, these exciting biological and technical advances compel us to revisit matrix degradation mechanisms in liver fibrosis regression. There is now the knowledge and know-how to address several key questions:

A. What are the cellular sites of expression of all known mammalian protease mRNAs and proteins in normal and injured liver? Surprises are sure to emerge. For example, interrogation of a human cirrhosis single cell transcriptomic dataset (61) reveals multiple cellular sources of TIMP-1 (data not shown), even though hepatic stellate cells had been defined as the only recognized source in earlier studies.
B. Does mRNA expression correlate with protein expression for proteases and their inhibitors?
C. What are additional functions or pathways regulating matrix protease and inhibitor expression, and which new therapeutic targets do they uncover?
D. Are pathways regulating matrix degradation conserved across tissues or are they unique to liver? Are these pathways the same in all liver diseases and stages of fibrosis?
E. What are the endogenous substrates and biologic outcomes of protease actions in normal and injured liver, and during progression or regression of fibrosis?
F. Where are proteases active within the normal and injured liver, and what are the cells and substrates most likely to be regulated by them?
G. Do proteases underlie the link between fibrosis regression and regeneration? The liver is remarkable in that healthy regeneration does not induce fibrosis, whereas advanced fibrosis prevents regeneration. Thus, what are the elements of fibrosis regression associated with cure of underlying liver disease that also lead to improved liver function, for example in patients effectively treated for hepatitis B or C?

These questions highlight the enormous unmet need and opportunity to advance our understanding of matrix degradation and its link to liver regeneration in human liver disease. Continued technological advances are certain to further facilitate the study of these important and under-explored questions.

5. Assessment of hepatic fibrosis

Histopathology has been a foundational tool in the diagnosis and management of liver disorders. Nonetheless, the assessment of liver fibrosis in chronic liver disease was merely descriptive until the early 1990s when the discovery of HCV and the launch of therapeutic trials for viral hepatitis led to the development of semi-quantitative scoring systems allowing the definition of different stages of disease progression (69, 70). These efforts concluded that fibrosis stage is the single most important predictor of significant morbidity and mortality. However, in clinical practice, quantification of fibrosis is still semi-quantitative and subjective. The introduction of computer-assisted morphometry, which allows quantitative and objective assessment of liver fibrosis, is an attempt to move beyond semi-quantitative systems. As emphasized above, the collagen proportionate area (CPA) (71) can accurately sub-classify cirrhosis and is the only validated predictor of clinical decompensation compared to all other histological sub-classification systems described to date (36). Despite this, semi-quantitative systems are still broadly used in clinical practice and clinical trials even though they are less appropriate than CPA. The advent of digital pathology that can now rely on scanning techniques for whole slide imaging will exponentially increase the speed and flexibility in evaluating digital histopathological images, and will finally facilitate the application of artificial intelligence (AI) with the creation of machine learning (ML) diagnostic algorithms (72). Recently, a ML-model based on trichrome-stained liver biopsy slides has been shown to predict CSPH in NASH patients with cirrhosis, for example (73).

Currently, elastography, ultrasound, computed tomography and MRI are the main diagnostic imaging modalities used in hepatology. In particular, the utility of MR-based biomarkers for the detection of features of NAFLD and its potential use in clinic or clinical research in NAFLD is advancing most rapidly. In this context, functional liver imaging may also accelerate the development of new treatments for NASH, especially when the mode of action of these drugs can be better reflected by changes in liver function. One of these modalities, the Gd-EOB-DTPA-enhanced MRI, is based on the use of the liver-specific MRI contrast agent gadoxetate disodium, which, when injected intravenously is taken up in hepatocytes via the organic anion-transporting polypeptide 1 (OATP1) transporter and excreted into the bile via the multidrug resistance-associated protein 2

(MRP2) transporter. In particular, Gd-EOB-DTPA-enhanced MRI has an excellent correlation with the histopathological assessment of liver fibrosis and its staging (74, 75).

Unmet needs

A. Validation of AI in analyzing data from digital imaging and pathology, coupled with electronic health records (EHR) will open a new era of precision medicine in hepatology that will progressively transform clinical practice. Ongoing efforts must overcome the inertia to resist change, and instead promote validation and adoption of these emerging technologies.
B. The roles of the hepatologist, the pathologist, and the radiologist must evolve as AI-based precision medicine is integrated into clinical care. How much these specialists should rely on these methods, and how can they be effectively leveraged to improve outcomes will be ongoing challenges.
C. The stratification of cirrhosis, currently largely based on surrogates, needs to based instead on precise biomarkers that more accurately quantify subtle changes in liver function, hemodynamics, immunity and inflammation, and regeneration. Such a multidimensional classification may incorporate molecular and cellular features in both liver and blood, coupled with imaging and functional readouts.

6. Non-invasive assessment of hepatic fibrosis

There is an acute need for methodologies to non-invasively assess hepatic structure and function with precision and reproducibility. Currently, regulatory agencies require liver biopsies to show evidence of benefit in phase 2B or phase 3 studies prior to potential approval of novel antifibrotic therapies. Yet, it is axiomatic that the liver biopsy must be replaced as a method to assess fibrosis, owing to its invasiveness, sampling variability, and the static nature of the information it yields. Moreover, a shrinking number of gastroenterologists and hepatologists perform biopsies, and therefore expertise among them is waning while biopsies are conducted instead by radiologists, at least in the United States.

Clinical trials in fibrotic diseases of other organs, for example lung, do not rely on tissue analysis, but rather on clinical, imaging, and functional readouts instead, and yet no organ has a richer functional repertoire than liver, one that is not sufficiently leveraged by the most widely used technologies. Furthermore, even approval of anti-fibrotic drugs based on biopsy improvement will be conditional, and require long-term evidence of improved clinical outcomes for full approval because biopsy is viewed as a 'surrogate' for the 'hard' endpoints of improving how a patient feels, functions or survives (i.e., clinical improvement)(76). Regardless, for as long as biopsies are required, efforts to validate highly quantitative digital methods to assess all elements of the biopsy (77, 78), combined with machine learning approaches (79), should be explored as quickly as possible, as emphasized in the preceding section.

Noninvasive fibrous diagnosis are evolving rapidly (80-83). The broad classes of non-invasive assessment tools include: 1) *Serum assays*, either using standard laboratory tests (e.g. FIB-4), proprietary assays for matrix components (e.g., ELF (84), ProC3/ProC5), proteomics (85), lipidomics (86), microRNAs (87, 88) and components of the microbiome (89); 2) *Imaging tests* (CT, MRI or PET) that can quantify liver fat, inflammation and fibrosis, as well as liver stiffness as a surrogate for matrix content (reviewed in (90, 91). Techniques for image collagen content directly using collagen-specific probes are also under development (92); 3) *Microbiome assessment*, since the pattern and diversity of the microbiome evolves with disease progression, for bacteria, viruses and fungi (93) (94-96); 4) *Functional tests,* which measure either intrahepatic shunting (97), microsomal activity (98, 99), or proteolytic activity either in liver or circulation (100).

Despite the substantial progress and significant investment in developing noninvasive markers of hepatic fibrosis, none has yet been able to supplant biopsy. There is a growing trend to combine existing tests or those under development, but it is too early to determine whether such efforts will reach the high standards of accuracy and predictive value required by regulatory agencies before biopsy can be abandoned as a measure of fibrosis content and response to therapy. Moreover, efforts like these are hamstrung by the requirement to correlate the results with biopsy, and thus their success is more likely to result from evidence that these noninvasive tests predict

clinical events, which will necessitate either long-term studies, or validation in patients who are at more imminent risk of complications (i.e., with more advanced fibrosis).

Unmet needs

In addition to the clear unmet need to replace biopsy, there are critical questions that must be addressed as non-invasive tests are further developed. These include:

A. What biological activities should we measure?

   i. *Fibrogenesis vs. fibrosis content?* Because there is a lag of unknown duration between changes in fibrogenic activity by myofibroblasts and their impact on fibrosis content in liver, greater effort should be invested in characterizing fibrogenic activity in hopes of providing a more sensitive and responsive readout of anti-fibrotic drug activity. Efforts of this type could include measures of binding activity by cell receptors expressed on fibrogenic cells (e.g., receptors for β-PDGF (101) or type VI collagen (102) , among others). While several of the widely used serum marker panels include assays for matrix molecules or their fragments, the mechanisms underlying their release into the blood and circulating levels are not well characterized, and thus their biologic plausibility is not fully established. On the other hand, specific imaging of hepatic collagen or other ECM constituents (103) may enable a more direct assessment of their content in liver.

   ii. *Functional reserve and regeneration?* As noted above, the liver is a functionally rich organ regulating myriad metabolic and homeostatic functions, with an unmatched secretome, and thus continued efforts to capture functional readouts of liver health and disease demand further investment. In cirrhosis, measures such as the MELD score, which clearly reflect function, robustly predict outcomes, yet it is unclear if subtle, overlooked features of hepatic function might be detectable at earlier stages – initial data using the HepQuant test hint that this may be possible (104). Efforts to catalog the proteome and lipidome in liver disease are promising, but candidate analytes typically lack biologic plausibility (ie., how do they contribute to liver disease and why do they change), which undermines interest in their development.

On the other hand, regression of fibrosis in patients cured of HCV or treated for HBV leads to improved liver function associated with regeneration, yet there has been almost no effort to capture regenerative signals, either by assessment of secreted molecules or by imaging.

iii. *Angiogenesis?* Angiogenesis is a vital part of liver repair, but also is a requirement for tumorigenesis, and the mechanistic distinctions between these two contexts are not clarified (105, 106). Still, quantitative assessment of angiogenic activity as a readout of hepatic healing or response to therapy has been overlooked.

B. Will different non-invasive markers be required for different stages of liver disease, or in different underlying diseases*?* It is quite likely that different stages of disease have distinct pathogenic drivers, especially in NASH where fat content declines with progression towards cirrhosis (107). Similarly, some features of fibrosis pathogenesis are distinct across different diseases (see above), and thus disease-specific markers are likely. A striking recent example is the immune micro-environment, which is distinct in NASH compared to other etiologies of liver disease, such that NASH patients with HCC are less likely to respond to checkpoint inhibitors (108). Similarly, subtle differences – not detectable by conventional histopathology or disease markers – may well underlie differences in fibrosis progression or regression. Indeed, early evidence indicates that among cirrhotics, fibrosis regression in NASH may be less likely than in other liver diseases (109), at least following bariatric surgery.

C. Should markers be used alone or in combination? The approach to combining different diagnostic modalities has been entirely empiric, seeking different combinations of blood, imaging, and functional tests to define the stage of liver disease and predict clinical outcomes. Yet, big data approaches using machine learning and artificial intelligence provide unparalleled opportunities to more rationally select therapeutic targets, define disease stage, and predict response to therapies (77, 110, 111). Of course, new algorithms using these approaches are only as good as the quality of data inputted, especially histologic features. Therefore,

validation of novel noninvasive algorithms should be based on highly quantified liver biopsy specimens using digital pathology strategies described above.

7. Target Discovery and Validation

Discovery of antifibrotic targets is a very vibrant field, as the evidence mounts for fibrosis regression and attenuated progression in response to disease-specific therapies in human liver disease (e.g., antivirals). The conventional and most widely used approach requires identification of molecules whose antagonism or agonism could influence fibrogenesis or matrix turnover based on their known biologic functions. Then, specific therapeutic candidates can be developed that are first tested in culture, then animals, and finally in humans if the drug has continued promise through these development stages. To identify potential targets, there is a rich and expanding variety of data available characterizing gene and protein expression both in public domain and proprietary data sets derived from rodent and human tissues and isolated cells, as described in the preceding section. In particular, single cell analysis has enabled the construction of comprehensive and detailed maps of fibrogenic cell heterogeneity not only liver, but other tissues as well (112-114). These remarkable tools not only illuminate the subtypes of myofibroblasts in different liver diseases, but also enable us to determine if candidate therapies targeting cells in liver might also affect other tissues, thereby reducing the possibility of unexpected off-target effects for liver-directed therapies. These massive and complex data sets also require the assimilation of new informatics skills to extract relevant information but can rapidly define potential targets based on their preferred cellular and tissue site(s) of expression, localization (cell surface, intracellular or circulating) and their association with specific biologic processes (e.g., secretion, stress responses, apoptosis, proliferation, and others).

In addition to these 'hypothesis-driven' approaches to identify targets based on the known functions of molecules in fibrosis, there are emerging computational and screening approaches that are unbiased and make no assumptions about what molecules might do in fibrotic diseases. Comprehensive approaches of this type can apply specific perturbations such as siRNA or CRISPR screens to define critical pathways or mediators whose inhibition reduces fibrogenic activity in

myofibroblasts. While widely utilized in uncovering novel cancer targets (115, 116), this approach can also be applied for fibrosis-related targets both in isolated cells and *in vivo* (117-119). Orthogonal approaches that integrate spatial gene expression, proteomics data, and assays that identify those gene targets that have open chromatin using ATAC-Seq can further leverage these unbiased approaches to target discovery. These technologies can be further strengthened by machine-learning approaches to refine target identification and validation and enhance the likelihood of clinical success (120).

Another unbiased approach, computationally based drug repurposing, has been validated in a number of diseases (121, 122), wherein large numbers of existing drugs are screened for their ability to reverse a disease gene signature in cultured cells, thereby identifying candidates with potential activity whose initial indication was unrelated to fibrosis (110). One of us (SLF) has used this approach to identify two candidate antifibrotic drugs despite no previous link to stellate cell biology or hepatic fibrosis (123, 124).

Complementing these approaches, potential targets may emerge from unbiased genome-wide association studies that link specific gene variants to disease outcomes. For example, a polymorphism in PNPLA3 is associated with risk of NASH not only through its effect on fat metabolism in hepatocytes, but also through a direct effect that enhances fibrogenic activity of hepatic stellate cells (125, 126). Because genomic variants are often discovered within previously unknown genes, these approaches can reveal novel biologic pathways, as was the case with the discovery of PNPLA3. Therapies may evolve directly from genomic information of this type by replicating the effect of protective variants or antagonizing disease-causing variants. One compelling example has been the identification of a variant in the PCSK9 gene that was protective against coronary artery disease, which immediately led to successful efforts to antagonize PCSK9 therapeutically (127). Similarly, there are now ongoing efforts to alter the function of PNPLA3 as a therapeutic strategy in NASH based on the biology of the disease-associated variant.

While there is no single ideal profile of an antifibrotic target expressed by activated stellate cells/myofibroblasts, some important features should include: *1) The target is easily accessible.* Cell surface receptors are most straightforward to engage, but methods are evolving to deliver

intracellular payloads to the stellate cell, for example using a liposomal delivery system containing vitamin A that delivers an shRNA within the cell (128); *2) Inhibition or alteration of the target does not interfere with normal stellate cell and liver function.* An appealing strategy is to target molecules that are only expressed by activated stellate cells/myofibroblasts, thereby minimizing any impact on homeostatic functions of stellate cells including vitamin A metabolism and support of hepatic regeneration. An early example is β-PDGF receptor, which is markedly induced during injury (129), but there are many others; *3) The target is most strongly or selectively expressed in injured liver*. While there is no single molecule that is absolutely stellate cell-specific, off target effects in other organs may be minimized by interrogating gene expression datasets to determine levels of expression of a candidate target in both normal liver and in other tissues. Also, drug delivery methods could contribute to identification of novel biomarkers to assess employ a dual targeting approach that requires engagement of two molecules that together may have far greater specificity. For example, stellate cells express a heterodimer comprised of angiotensin II Type 1 receptor combined and the type 1 cannabinoid receptor (130) whose expression may be more restricted to liver.

The pharmacology of potential therapeutics will influence their appeal as a treatment for liver fibrosis. These features include the route of administration, frequency, tolerability, drug-drug interactions and safety. While small molecules are most attractive because they can be administered orally, some larger biologic agents including antibodies and nucleic acid formulations can be administered infrequently – sometimes monthly or even less often, narrowing the advantage of small molecule therapies.

Testing of candidate antifibrotic therapies may progress from simple to more complex systems, and from small animals to non-human primates prior to human clinical testing. The more representative a testing platform is of its *in vivo* behavior in human liver disease, the higher the level of confidence in its potential efficacy as a drug. For direct acting molecules intended to attenuate stellate cell fibrogenesis, both primary and immortalized mouse and human stellate cells are a robust initial validation tool, provided that they express the relevant target molecules similar to their expression *in vivo*. More recently, generation of hepatic stellate cells from induced pluripotent stem

cells offers the prospect of replicating specific genetic backgrounds within the cells that may contribute to disease risk, pathogenesis and/or fibrogenic activity (131).

Culture models for testing for antifibrotic agents are increasingly sophisticated in an effort to better mimic the physical, chemical and intercellular properties of the human fibrotic liver. These approaches include single cell or multi-cellular organoids (132), precision cut liver slices from normal or diseased rodent and human liver (133, 134), or using substrata of varying stiffness (135). In addition, artificial liver devices ("Liver on a Chip") seek to systematically replicate all cellular, mechanical and soluble elements of the human liver using defined components in highly reproducible platforms (136, 137); these technologies are further optimized through use of 3D printing (138). A related technology, as noted above, is the use of extracellular matrix 3D scaffolds to more faithfully test the impact of materials derived directly from human liver on drug responses (22).

A detailed discussion of animal models to test antifibrotic drugs is beyond the scope of this article and has been the subject of recent reviews, focused especially on NASH (139, 140). Nonetheless, key elements of any model testing antifibrotic drugs should include the following: 1) The disease pathogenesis and behavior of fibrogenic cells should mimic human disease; 2) Specific candidate therapeutic targets should be expressed by the same cells and at levels comparable to human disease; 3) Efficacy should be validated in more than one model that are mechanistically distinct; 4) Drugs should be efficacious when administered after disease is already established, similar to the clinical setting, rather than solely preventive; 5) The drug's pharmacology should be similar between rodents and humans; 6) There should be evidence of target engagement, that is, that the effect of a drug should be directly attributed to interaction with its intended target and not through an off-target activity.

While rodent models of hepatic fibrosis have been a mainstay of drug testing for decades, there is increasing frustration with their relevance and translation to human drug efficacy, especially since no drug has yet been approved for hepatic fibrosis despite many promising prospects based on animal studies. This issue has been addressed in recent reviews (141, 142), and here we emphasize three key points: *1) The potential contribution of the microbiome.* Recent elegant

studies demonstrate that the composition of the microbiome can influence the efficacy of drugs in animal models, such that use of animals with a more complex wild mouse microbiome more faithfully predict subsequent outcomes in human trials, although this finding has not yet been extended to studies testing antifibrotic drugs; *2) Short duration of disease in animals.* Human liver disease evolved over decades, allowing for progressive cross-linking of collagen that makes it more insoluble, and distortion of the hepatic architecture. These critical features may not be fully replicated in the relatively short interval used for rodent models that test anti-fibrotic drugs; *3) Because multiple pathways drive NASH therapies targeting only a single pathway or molecule may not be sufficiently active*. A recent study has defined networks of gene expression that together stimulate fibrosis in NASH (143), implying that disruption of multiple pathways may be required to achieve a therapeutic benefit; 4) *NASH may be comprised of different subtypes that have distinct disease drivers, and therefore different therapeutic targets*. Disease subtypes, also called endophenotypes (144), have been described in both diabetes (145) and NASH (146), but have not yet been exploited to enrich clinical trials with specific patient subgroups.

Unmet needs

A. Harness powerful new technologies to identify and optimize therapeutic targets and identify novel biomarkers. The time is right to leverage single cell technologies and drug repurposing algorithms, powered by artificial intelligence, to refine candidate therapeutic targets. These efforts should be complemented by deeper analysis of human liver samples from patients in clinical trials - both of those who respond as well as non-responders - to identify what markers correspond with a therapeutic benefit. Such efforts well not only uncover unexpected molecular drivers of efficacy, but also identify candidate biomarkers of fibrotic content or activity that could yield new non-invasive diagnostics.

B. Streamline and validate robust pipelines for pre-clinical drug testing. Current approaches are time-consuming, and overly optimistic in predicting drug efficacy using rodent models of disease. Refinements in these animal models, possibly by manipulating the microbiome, can be complemented by more sophisticated organoid and other

complex ex vivo models, translating predictors of efficacy derived from human tissues into these higher throughput pipelines.

C. For studies that rely on biopsy, validate digital pathology methods (see above). The rich dataset generated by these methods establishes a strong rationale to assess their utility in quantifying response to therapies and predicting clinical events. These efforts will require access to large numbers of liver biopsies from longitudinal cohorts.

D. Develop data-driven rationales for combination therapies. Clinical trials to date using single drugs in NASH fibrosis have shown disappointing efficacy, which has provoked efforts to try combination therapies that attack different targets underlying disease pathogenesis. However, the choice of combinations has been entirely empiric, and often driven by which drugs are available to a commercial sponsor. Efforts should be accelerated to leverage pre-clinical or high throughput systems (e.g, organoids) in assessing global transcriptomic or proteomic responses to large numbers of combinations. Those combinations that are synergistic can be further evaluated using *in vivo* models of disease.

E. Seek characterization of subtypes of disease that may more responsive to antifibrotics. Currently only the fibrosis stage is used to select patient subgroups for treatment trials, yet there may be specific targets that define subgroups based on both genetics and gene expression data. Accurate identification of such patients could greatly enrich clinical trials and improve treatment responses.

8. Clinical Trials of Antifibrotics

Almost all current and planned clinical trials targeting fibrosis are focused on NASH, which has been the subject of several reviews (40, 147-149). Among the antifibrotic targets in NASH, several specifically target stellate cell activation and/or fibrogenesis, however none is yet approved, and thus are not reviewed in detail here. The general approaches to antifibrotic therapies can be subdivided among the following: 1) agents directly targeting hepatic stellate cells or their components, including fibrogenic, proliferative, apoptotic and/or contractile

molecules, as well as cell based strategies to ablate senescent stellate cells: 2) drugs that modify inflammation or injury to reduce fibrogenic signals that activate stellate cells; 3) molecules that target extracellular matrix structure and cross-linking to reduce its stability and accumulation; 4) agents that provoke matrix degradation, either by amplifying cells driving proteolysis, and/or unmasking proteolytic activity, for example by inhibiting tissue inhibitors of metalloproteinases (TIMPs). Among these, the most advanced efforts have been direct stellate cell therapies, modifiers of inflammation and injury, and inhibitors of collagen cross-linking, but none have yet shown efficacy in human trials. Approaches outlined in the preceding section will continue to refine these targets and seek their efficacy in pre-clinical models and early clinical trials.

From a clinical trial perspective, three key issues in assessing antifibrotic therapies are: what to measure, how to measure it and how long should trials be conducted? As detailed above, the field is held back by the lack of robust non-invasive markers of fibrosis and fibrogenesis that can assess efficacy at progressive intervals without biopsy. For NASH, most trials are 6 – 18 months, but this interval is largely empiric. Natural history studies in hepatitis B and C, as well as NASH suggest that some fibrosis reduction is possible in a year or less, but is greater at 5 years (109, 150, 151). Moreover, whereas reversal of cirrhosis has been documented in HBV and HCV, this has not been consistently observed in NASH cirrhosis. Unfortunately, animal models are not very informative in this regard, as the entire disease process that takes decades in humans is telescoped into weeks to months in rodents.

The goals and targets of antifibrotic therapy in liver will also need to account for the stage of disease. Patients with intermediate stages of fibrosis may benefit clinically from simply attenuating progression, whereas those with cirrhosis will more likely benefit if a therapy induces matrix degradation and provokes hepatic regeneration with improved synthetic function. Currently, trials divide patients between cirrhosis and non-cirrhosis, but this is a blunt distinction and will benefit from greater clarity about the point at which fibrosis or cirrhosis is no longer reversible, i.e., ‘the point of no return’.

Progress in the broader field of antifibrotic therapies has also been disappointingly slow. Given the liver's unique regenerative capacity, most chronic diseases, including NASH, require decades to accumulate sufficient scar in order compromise organ function, whereas in lung, for example, the disease carries significant mortality within 3-5 years. Thus, there may be greater prospects for antifibrotics' success in liver than other tissues. Yet only pulmonary fibrosis has two approved drugs, pirfenidone and nintedanib, but these only delay the rate of deterioration and are difficult to tolerate due to adverse effects (152), and there are no approved antifibrotic therapies for any other organ or condition. Nonetheless, the pace of progress in understanding disease pathogenesis and regulation of fibrosis has been accelerating, and there is little doubt that the next few years will translate these advances into effective therapies.

Unmet needs

A. Clarify the rates of fibrosis regression in different liver diseases at different stages to refine trial durations and endpoints. Trials in patients with more advanced fibrosis or cirrhosis may be less 'regressible' and require more time to achieve clinically meaningful benefit.

B. Explore the prospects for antifibrotic trial enrichment by better defining subgroups of patients using genetic, molecular and non-invasive markers. While reaching this goal is not imminent, clarification of disease drivers and targets in different groups of patients will enhance the prospects for more conclusive clinical trials, even if this means drugs may be approved for smaller, better-defined patient cohorts.

C. Design clinical trials that include hard endpoints, where possible. Because an improvement in fibrosis is a surrogate endpoint that may not translate into clinical benefit, either longer trials and/or those using markers that directly predict clinical outcomes with have greater appeal to regulatory agencies, providers and patients.

The ongoing exploration of hepatic fibrosis has borne tremendous fruit in defining key cellular and molecular determinants of chronic liver disease. These advances have led to sustained optimism that fibrosis will yield to effective antifibrotic therapies that improve the lives of patients

with chronic liver disease. Success will surely come, but there is more work to be done to address unmet needs (Figure 2). We hope this article will help streamline progress and fertilize the field in the coming years.

Figure Legends

Figure 1. Different patterns of fibrotic evolution in different chronic liver diseases. The three major patterns of fibrotic evolution are illustrated. The "post-necrotic" pattern is typical of chronic viral hepatitis and is mainly characterized by porto-central evolution with early involvement of the centrilobular vein (CLV). Sinusoidal arterialization and neo-angiogenesis are also typical of this pattern. Fibrogenesis originating in lobular zone 3 ("pericentral fibrosis") is a main feature of chronic alcoholic hepatitis and non-alcoholic steatohepatitis (NASH). Here the main feature is the capillarization of sinusoids in zone 3 that progressively becomes panlobular. "Biliary fibrosis", a key feature of primary biliary cholangitis and primary sclerosing cholangitis, evolves mainly portal to portal with progressive worsening of cholestasis.

Figure 2. Current unmet needs in hepatic fibrosis from basic to translational to clinical perspectives. Shown are the unmet needs and current gaps in hepatic fibrosis outlined in this review, surrounding an image of a cirrhotic liver within which are the cellular changes on the left, and the appearance of fibrosis on the right. These unmet needs are displayed along the continuum from basic, through translational, to clinical research.

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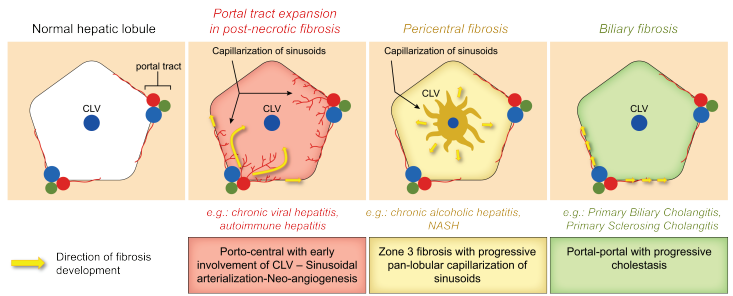
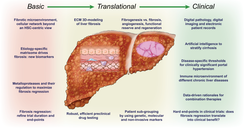
Figure 1


Normal hepatic lobule
Portal tract expansion in post-necrotic fibrosis
Pericentral fibrosis
Biliary fibrosis
portal tract
CLV
Capillarization of sinusoids
CLV
Capillarization of sinusoids
CLV
CLV
e.g.: chronic viral hepatitis, autoimmune hepatitis
e.g.: chronic alcoholic hepatitis, NASH
e.g.: Primary Biliary Cholangitis, Primary Sclerosing Cholangitis
Direction of fibrosis development
Porto-central with early involvement of CLV – Sinusoidal arterialization-Neo-angiogenesis
Zone 3 fibrosis with progressive pan-lobular capillarization of sinusoids
Portal-portal with progressive cholestasis


Basic
Translational
Clinical
Fibrotic microenvironment, cellular network beyond an HSC-centric view
Etiology-specific mechanisms drives fibrosis: new biomarkers
Metalloproteases and their regulation to maximize fibrosis regression
Fibrosis regression: refine trial duration and end-points
ECM 3D-modeling of liver fibrosis
Robust, efficient preclinical drug testing
Fibrogenesis vs. fibrosis, angiogenesis, functional aspects of vascularization
Patient sub-grouping by using genetic, molecular and non-invasive markers
Digital pathology, digital imaging and electronic patient records
Artificial intelligence to stratify cirrhosis
Disease-specific thresholds for clinically significant portal hypertension
Immune microenvironment of different chronic liver diseases
Data-driven rationales for combination therapies
Hard end-points in clinical trials: does fibrosis regression translate into clinical benefit?