Review

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Hepatic fibrosis: Concept to treatment

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Summary

Understanding the molecular mechanisms underlying liver fibrogenesis is fundamentally relevant to developing new treatments that are independent of the underlying etiology. The increasing success of antiviral treatments in blocking or reversing the fibrogenic progression of chronic liver disease has unearthed vital information about the natural history of fibrosis regression, and has established important principles and targets for antifibrotic drugs. Although antifibrotic activity has been demonstrated for many compounds *in vitro* and in animal models, none has been thoroughly validated in the clinic or commercialized as a therapy for fibrosis. In addition, it is likely that combination therapies that affect two or more key pathogenic targets and/or pathways will be needed. To accelerate the preclinical development of these combination therapies, reliable single target validation is necessary, followed by the rational selection and systematic testing of combination approaches. Improved noninvasive tools for the assessment of fibrosis content, fibrogenesis and fibrolysis must accompany *in vivo* validation in experimental fibrosis models, and especially in clinical trials. The rapidly changing landscape of clinical trial design for liver disease is recognized by regulatory agencies in the United States (FDA) and Western Europe (EMA), who are working together with the broad range of stakeholders to standardize approaches to testing antifibrotic drugs in cohorts of patients with chronic liver diseases.

Introduction

After acute injury, the liver can restore its complete mass and original architecture in a relatively short interval even when a large fraction of the organ is destroyed. In contrast, chronic liver injury, as triggered by different etiologies, induces repetitive tissue damage, resulting in impaired regenerative capacity marked by an altered inflammatory infiltrate and a chronic wound healing response [1,2]. The response to chronic injury also includes necrosis and/or apoptosis of parenchymal cells and their replacement by extracellular matrix (ECM). Although initially beneficial, the wound healing process becomes pathogenic if it progressively replaces parenchyma with scar tissue and distorts the liver vascular architecture, eventually resulting in organ dysfunction.

To date, specific therapies for liver disease have primarily been etiology-driven by eliminating or ameliorating the causative agent of chronic liver diseases (CLD). Recent examples are the spectacular successes in blocking replication and/or curing chronic hepatitis B and C virus infections, which have built upon molecular insights into cellular infection and viral replication. These strategies have been fruitful in blocking liver injury and thus progression of fibrosis, and even in reversing advanced fibrosis. However, findings in both human studies and animal models nicely emphasize that liver fibrosis is a dynamic process that can be modulated either by halting progression and/or promoting resolution. Thus, the clarification of molecular mechanisms underlying these events is fundamentally important for establishing antifibrotic therapies. The increasing success of antiviral treatments in blocking or reversing the fibrogenic progression of CLD has unearthed vital information about the natural history of fibrosis regression, and has established important principles and targets for antifibrotic drugs.

Pathophysiology: established and emerging mechanisms

The progression and resolution of fibrosis is a complex process involving parenchymal and non-parenchymal liver cells, as well as infiltrating immune cells. Chronic hepatocyte death via apoptosis, necrosis or necroptosis is a critical step. Cell death induces activation of inflammatory and pro-fibrogenic pathways in non-parenchymal cells and infiltrating immune cells, which trigger fibrosis progression, but may also contribute to fibrosis resolution [1–3].

The fibrogenic response, as characterized by scar formation due to increased production and deposition of ECM proteins, is

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the essential step that culminates in major changes in liver architecture. Modifications in ECM composition and content not only have mechanical and physical consequences, but also contribute to the modulation of cellular functions such as growth, migration, and gene expression, in part through the direct interaction between ECM components and cell adhesion molecules. The ECM also functions as a reservoir for pro-inflammatory and pro-fibrogenic mediators [4].

The key fibrogenic effector cell type in the liver is the activated hepatic stellate cell (HSC), although other cells and processes can make significant contributions. HSCs are characterized by the ability to store retinyl esters in intracytoplasmic lipid droplets, and by ultrastructural features of vascular pericytes consistent with their role in regulating sinusoidal blood flow [5]. The features of HSC activation and their phenotypic transformation into myofibroblasts, as well as their pro-fibrogenic role, have been extensively clarified and represent an important basis for the understanding of hepatic fibrogenesis [1–4]. The transition of HSCs into myofibroblasts is regulated by their interaction with several cell types and the activation of specific pathways that are framed within the context of the wound healing reaction (Fig. 1). Besides injured hepatocytes, hepatic macrophages, endothelial cells, and lymphocytes drive HSC activation. The death of hepatocytes leads to the release of cellular contents (e.g. DNA and damage-associated molecular patterns known as DAMPs) and reactive oxygen species that activate resident macrophages (Kupffer cells) to release pro-inflammatory factors like TNFα, IL-1β, and IL-6, and pro-fibrogenic factors, especially TGFβ. Additional pro-inflammatory factors include chemokines like CCL2, as well as gut-derived pathogen-associated molecular patterns (PAMPs). An example is the activation of toll-like receptor 4 (TLR4) that leads to repression of the activin membrane bound inhibitor BAMBI, which further enhances TGFβ-dependent HSC activation [6,7].

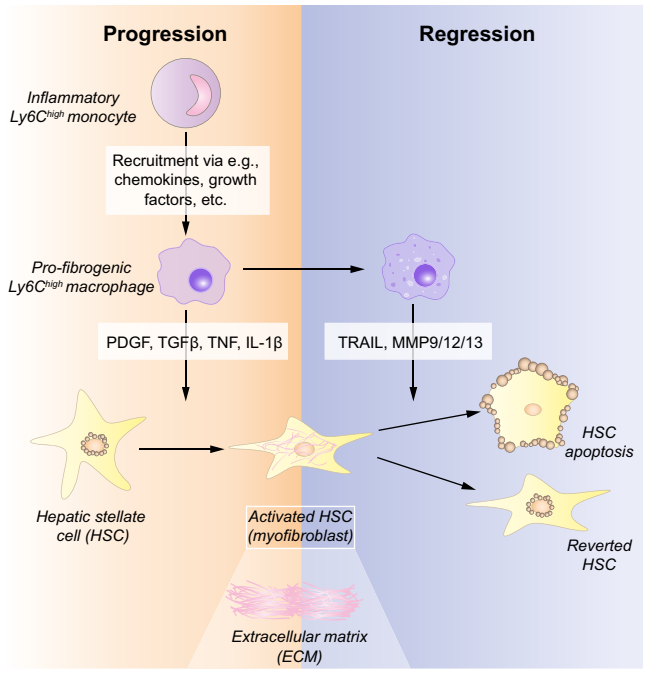

Fig. 1. Macrophages are the fuel and brake of liver fibrosis progression and resolution. The cartoon illustrates the interplay between macrophages and hepatic stellate cells (HSCs) during liver fibrosis progression and resolution. Acute or chronic hepatocyte injury triggers the recruitment of inflammatory (Ly6C^{hi}) macrophages into the liver. Persistently activated Ly6C^{hi} macrophages during chronic injury are a driving force via soluble factors e.g. growth factor, cytokine and chemokine to induce the transition of resting HSCs into activated HSCs (myofibroblasts; MFBs). MFBs continuously produce extracellular matrix (ECM) leading to liver scarring. If chronic injury can be stopped e.g. by eliminating HCV, macrophages will change their phenotype from an inflammatory to a pro-resolution Ly6C^{low} phenotype. Ly6C^{low} macrophages lose their ability to stimulate and maintain the MFB phenotype. As a consequence MFBs either revert to HSCs or die from apoptotic cell death. ECM production is stopped and over time ECM is resolved.

In addition to chronic wound healing, oxidative stress contributes to all fibrogenic disorders characterized by chronic tissue damage, and to the overexpression of critical genes related to extracellular matrix remodeling and inflammation [8]. Oxidative stress resulting from the activity of free radicals, as well as by decreased efficiency of antioxidant defenses is not simply a toxic consequence of chronic tissue injury, but rather actively contributes to excessive tissue remodeling and fibrogenesis, especially in alcoholic hepatitis and non-alcoholic steatohepatitis (NASH) [9].

More recently, attention is progressively shifting towards the pro-fibrotic microenvironment of the liver, with increasing interest in the role of immune cells, and especially subsets of macrophages, in regulating the progression or the regression of fibrosis (Fig. 1) [10]. During acute injury, Kupffer cells (hepatic macrophages) coordinate the regenerative response. However, during chronic injury, Kupffer cells drive fibrosis progression, since they not only activate HSCs but also stimulate the influx of bone marrow derived immune cells via release of CCL2 and CCL5 [10]. Thus, the recruitment of immature monocyte-derived Ly6C^{hi} macrophages is dependent on CCL2 secreted by Kupffer cells and HSCs [11,12]. In murine models, Ly6C^{hi} macrophages promote fibrosis progression because their deletion (e.g. in *Cd11b-DTR* transgenic mice), inhibits the pro-fibrogenic response in a model of carbon tetrachloride (CCl_4) induced fibrosis. Hence, immature Ly6C^{hi} CD11b^{+}F4/80^{+} macrophages and their CCL2-dependent accumulation are a central mechanism of fibrosis activation and progression [13]. However, inflammatory and pro-fibrogenic Ly6C^{hi} macrophages can differentiate into pro-resolution (restorative) Ly6C^{lo} macrophages. Pathways underlying this switch are of intense interest, because they convert the fibrogenic microenvironment to one that promotes resolution of liver fibrosis. The fractalkine receptor CX_3CR1 may be a key pathway mediating this switch, because its greater abundance is associated with a pro-resolution Ly6C^{lo} macrophage phenotype [14]. While the switch of the less mature pro-inflammatory Ly6C^{hi} to the mature pro-resolution Ly6C^{lo} macrophage is therapeutically appealing, it has proven difficult to accomplish *in vivo*. Ly6C^{lo} macrophages secrete larger quantities of candidate fibrolytic matrix metalloproteinases such as MMP-9 and MMP-13, and the anti-inflammatory cytokine IL-10, which are all implicated in fibrosis resolution. The relevance of this concept has been supported by the finding that transfer of restorative Ly6C^{lo} macrophages reduces liver scarring in CCl_4-induced liver fibrosis.

Other major areas of development include the role of intestinal microbiota [15,16], the role of tissue hypoxia [17] with the establishment of an anaerobic pro-inflammatory environment [18], the influence of epigenetic modification in conditioning the progression of fibrosis [19] and the weight of tissue stiffness on the progression of the fibrogenic process [20].

Fibrosis reversibility: the ultimate target

The prospect of fibrosis reversibility is striking, and trials using antiviral drugs have established a clear proof of concept for this possibility in humans. Animal models have identified HSCs as an important molecular target. Gliotoxin administration specifically induces apoptosis of HSCs to reverse fibrosis *in vivo*. Recent studies employing fate tracing experiments to monitor the plasticity of HSCs have improved our understanding of HSC behavior *in vivo* during fibrosis resolution. HSCs, following their activation to myofibroblasts, can revert to an inactivated, albeit not fully quiescent, state [21]. During fibrosis resolution some myofibroblasts revert into inactivated HSCs, while the remaining myofibroblasts are triggered to become apoptotic – the fraction of HSCs that follow each of these fates is not yet clear. Most likely, the lack of pro-survival signals in a non-fibrogenic liver environment contributes to HSC reversion or apoptosis, however the master switches to push myofibroblasts towards HSC reversion or death are not known either. Interestingly, reverted HSCs are more prone to myofibroblast transdifferention after subsequent pro-fibrogenic insults, indicating that a previously injured liver is more susceptible for new insults because 'inactivated' HSCs are more easily re-activated to become fibrogenic. Despite the documented evidence of fibrosis and cirrhosis regression in animal models and the reabsorption of scar tissue following an effective primary treatment in humans (i.e. sustained viral response, abstinence from alcohol etc.), the full reversibility of fibrosis in patients with CLD for 30 years or more is still debated. Indeed, at advanced stages of the disease, scar tissue is marked by extensive collagen cross-linking, with a greater presence of elastin, dense acellular/paucicellular ECM, and decreased expression and/or activity of specific metalloproteinases [22,23]. In addition, long-term fibrogenesis in human CLD is characterized by a progressive resistance to apoptosis of HSC/myofibroblasts, leaving a critical mass of pro-fibrogenic cells refractory to reversion back to a quiescent state [24].

More recent research has focused on the biochemical changes affecting fibrosis irreversibility. An important advance has been the identification of lysyl oxidase 2 (LOXL2) in catalyzing the cross-linking of extracellular collagens [25]. LOXL2 stabilizes the ECM, and in more advanced stages reduces fibrosis reversibility. Therefore, advanced liver cirrhosis may become increasingly irreversible, but we do not know if there exists a critical point of virtual irreversibility. Moreover, future new treatment options will eventually demonstrate to what extent we can reverse cirrhosis therapeutically.

Target selection, multicellular approach and combination therapy

Activated HSCs, (portal) myofibroblasts and the ECM that they produce are primary targets of antifibrotic therapies. However, HSC and myofibroblasts communicate with numerous other cell types that can promote their fibrogenic activation, induce their quiescence and apoptosis, or remove excess ECM via release of fibrolytic enzymes and phagocytosis; together these represent additional, complementary pharmacological targets. Agents that target HSC, myofibroblasts and the ECM are 'direct antifibrotics', whereas therapies that address the other cells and pathways are 'indirect antifibrotics'.

For devising antifibrotic therapies it is useful to consider two major multicellular functional units that contribute to fibrosis with a varying extent, depending on the etiology of liver disease and its stage: 1) *perisinusoidal/vascular* – with pericytes, i.e. HSC, sinusoidal endothelial cells, macrophages/Kupffer cells, other inflammatory cells, and hepatocytes; 2) *portal/periportal* – with cholangiocytes/ductular cells, portal fibroblasts and myofibroblasts, and various inflammatory cells; this unit also includes a stromal inflammatory compartment – with fibroblasts and myofibroblasts, T and B cells, and macrophages/dendritic cells [26–28].

The cellular interactions within these units also define a spectrum of growth factors, cytokines, and proteases that serve as targets for antifibrotic therapies (Fig. 2). In fibrosis, the cellular interaction within and between these multicellular units is skewed towards a chronic wound healing response by excess deposition of scar tissue and vascular remodeling, a default mechanism that is aimed at maintaining tissue integrity under continuing (inflammatory) injury, at the expense of functional parenchymal tissue. Notably, even advanced fibrosis/cirrhosis can reverse to a normal liver architecture when the injury is eliminated, as has been shown impressively in patients on effective treatment of chronic hepatitis B and C [29,30]. This process is too slow for many patients with advanced fibrosis and often no causal therapy is available, necessitating the development of antifibrotic therapies that are both effective and free of side effects. Such therapies should carefully modulate the multicellular units towards fibrolysis and direct them towards the original set-point of non-fibrotic tissue maintenance. To this end combination therapies that address two or more key cellular or molecular players and/or pathways will be needed.

With the current tools of drug development and validation it is difficult to realize such a combination approach. First, safety and efficacy testing of a single agent, even with an optimal biopsy-based study design, will require several hundred patients, last many years and be very costly, with a significant risk of failure [31]. Second, the combination of two agents with proven efficacy and safety would require another round of clinical validation. Third, different companies may rather prefer to develop and market a monotherapy with limited but proven efficacy than investing a huge additional effort in validating a promising combination therapy, especially when the second drug is owned by another company. A more optimistic view, however, could envision a paradigm similar to cancer therapeutics, where combination therapies and cooperation among commercial stakeholders have become the rule rather than the exception.

To accelerate the preclinical development of potent antifibrotic combination therapies, a reliable single target validation is necessary, followed by a rational selection and testing of combination approaches. Improved noninvasive tools for the assessment of fibrosis content, fibrogenesis and fibrolysis must accompany *in vivo* validation in experimental fibrosis models, and especially in clinical trials. Therefore, a top priority is the development of biomarkers that can be determined frequently during the course of a treatment (serum parameters and/or imaging) and that truly reflect the underlying pathogenesis that is targeted by medications [26,27,31–35]. In liver fibrosis, such markers would quantify the extent of fibrogenesis and/or fibrolysis (ECM deposition and removal, respectively) before and during treatment, and permit an individualized dose adjustment of a mono- or combination therapy. Moreover, under the assumption

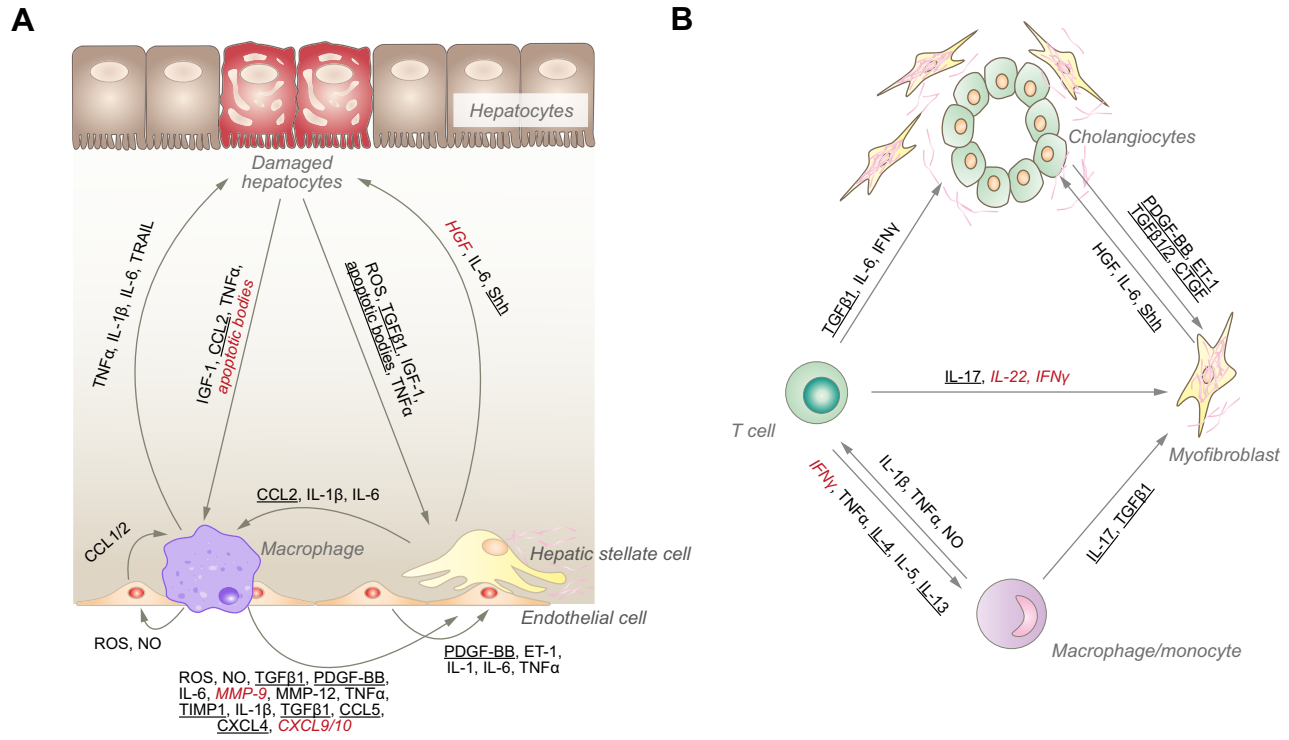

Fig. 2. Targeting the multicellular context of fibrosis. Major functional units and secreted factors to be addressed by antifibrotic therapies. (A) Vascular and (B) biliary and interstitial unit. Pro-fibrogenic targets are underlined, in contrast to putative fibrolysis-inducing targets in italics and red. Select examples are discussed in the text. Modified from [26–28]. CCL, CC chemokine ligand; CTGF, connective tissue growth factor; CXCL, CXC chemokine ligand; ET-1, endothelin-1; HGF, hepatocyte growth factor; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; MMP, matrix metalloproteinase; NO, nitric oxide; PDGF-BB, platelet-derived growth factor BB (recent data show that most of PDGF-BB in liver fibrosis derives from activated platelets [38]); ROS, reactive oxygen species; TNFα, tumor necrosis factor α; Shh, sonic hedgehog; TGFβ1, transforming growth factor beta1; Th, T helper cell; TIMP, tissue inhibitor of metalloproteinases; TRAIL, TNF-related apoptosis-inducing ligand; Treg, regulatory T cell.

that the level of the serum marker or the intensity of the imaging signal over time correlates with the final extent of fibrosis, the stratification of patients could be improved, and the needed number to be tested and the duration of clinical studies reduced dramatically. Such biomarkers ideally should correlate with the effects on morbidity and mortality due to decompensated cirrhosis or HCC. Finally, such markers could pave the way for a true personalized medicine, where different patients might receive different combinations and doses of antifibrotics, adjusted by relying on the predictive biomarkers. While there have been some advances in biomarker development and validation, further improvement remains a top priority to optimize and guide antifibrotic drug development [26,27,32–34]. Notably, the availability of valid biomarkers of fibrogenesis could establish the antifibrotic activity of long-used inexpensive and relatively safe medications, such as statins [36], or aspirin, which prevents the release of pro-fibrogenic PDGF-BB in models of liver fibrosis progression and reversal [37]. By targeting different fibrogenic pathways, combinations of repurposed drugs could be more efficient as the side effect profiles would be known, and there would no need for phase 1 trials to establish safety.

Preclinical proof of concept

The antifibrotic effect of a large number of compounds has been demonstrated *in vitro* and in animal models of liver fibrosis over the past two decades. Numerous compounds seem to have an adequate safety profile in animal models and in phase 1 clinical studies, or alternatively the drug is an existing agent used for other clinical indications, and is re-discovered as an antifibrotic ("drug repositioning") [26,34]. However, none of these repurposed drugs have been thoroughly validated in the clinic or commercialized as a therapy for fibrosis. An uncertainty is the translation of the wealth of information on antifibrotic agents derived from *in vitro* and *in vivo* animal experimental studies into meaningful advances for patients. A major difficulty in translating animal studies to human diseases is the complexity of the interactions between cells, soluble mediators, the ECM and its receptors (i.e. the pro-fibrogenic microenvironment), and intracellular signaling relevant to the fibrogenic process. In this context, most of the information gathered is 'mono-mechanistical'; i.e. each study highlights the role of one cell, one cytokine, one receptor, or one signaling molecule, without considering that the results obtained are just a very reductionist view of the complex process of hepatic fibrogenesis.

In vitro models

Currently, biologic targets for antifibrotic therapies are identified by studies done in 2D monolayer single cell cultures or co-cultures (activated HSC and other liver cells that contributed to fibrogenesis or fibrolysis) on plastic dishes followed by a "validation" in mouse or rat models. In this context, it is important to

highlight that the identification of fibrogenic targets following activation of HSC in 2D monolayer single cell cultures only partially corresponds to the pattern of target activation found in animal models of liver fibrogenesis [38]. Drug development is based on this methodological sequence, and newly developed drugs are tested on the same *in vitro* and *in vivo* models, and then often proposed for human use. In part, the current lack of effective translation is due to the inherent problems associated with drug evaluation in suboptimal models. For example, 2D cell cultures of hepatic fibrogenic cells (HSC) exhibit constitutive activation of a "myofibroblast-like" phenotype following growth on an artificial plastic substrate. Importantly, cell culture plastic surfaces mimic a tissue tension of 10^6 kPa compared to tension of the liver 3D structure, which ranges between 5 (normal liver) and 20 (fibrotic/cirrhotic liver) KPa [39,40]. Thus, conventional 2D cell culture is suboptimal as a surrogate to test and evaluate drug targets. The introduction of *in vitro* systems for target discovery and drug screening that more faithfully replicate the pro-fibrogenic microenvironment of human liver is greatly awaited. These models should at least ensure the presence of a 3D structure and the expression of a sufficient physiological and pathophysiological variety of ECM components. Indeed, there is increasing evidence of the different effect of 2D and 3D ECMs on key biological features of fibroblasts and myofibroblasts, including proliferation, migration, contraction, matrix deposition and degradation [41,42]. Of particular interest is myofibroblast mechano-function, and different models have been generated to mimic normal skin, wound repair, tissue morphogenesis and remodeling, as well as growth and contracture during scarring/fibrosis [43,44]. Recent data obtained by culturing human HSC in ECM scaffolds from decellularized human liver tissue have highlighted remarkable differences in gene and protein expression of established pro-fibrogenic agonists/pathways, when compared to standard 2D cultures on plastic dishes [45]. Accordingly, using 3D *in vitro* cultures or co-cultures as an initial methodological step may be a better initial approach to identify pharmacological targets. Following *in vivo* validation in adequate animal models, bioengineered human 3D ECM scaffolds could then represent the next step in verifying drug efficacy before testing in human trials.

Animal models of liver fibrogenesis

Improved animal models are required to assess antifibrotic efficacy within the complex multicellular context of disease, and to study the bioavailability, pharmacokinetics, and toxicity of candidate antifibrotic agents [33]. *Mdr*2 KO mice that lack the hepatocyte phospholipid flippase Mdr2 mimic human primary sclerosing cholangitis as a model of spontaneous biliary fibrosis progression (*Mdr*2 KO) [46], while administration of toxins (CCl_4 and/or thioacetamide) induces a progressive parenchymal fibrosis, which after long-term administration, shows little reversibility if the agent is discontinued [47]. Drugs that work in multiple models will have a higher likelihood to be effective in human fibrosis, which is less inflammatory and necrotic than most animal models. Although (mono-) genetic models (e.g. transgenic mice with overexpression of PDGF-B, PDGF-C, or TGFβ1 [48–50]) can validate factors and mechanisms that are central to fibrosis, transgene expression in these models is either largely ectopic (hepatocytes) or disregards regulatory circuits operative in non-transgenic fibrotic animals. Importantly, *in vivo* models should be performed according to standardized guidelines. These include: 1) selection of animals of defined (adult) age and sex; 2) group sizes of 8–15 animals; 3) optimized route and dose of toxin application; 4) analysis of liver samples of sufficient size (5–10% of the organ); 5) representative morphometric analyses; 6) the use of a representative spectrum of quantitative fibrosis and fibrolysis readouts [33]. Many published studies do not satisfy these criteria.

Precision-cut liver slices (PCLS)

Transferability of animal studies to the human situation is uncertain and may vary with the pharmacological target. Human PCLS are ~200 μm thick sections of liver that can be cultured for several days and partly reflect the multicellular human context [51]. They can be isolated from normal liver resections, which undergo spontaneous fibrogenic activation in culture, or from cirrhotic liver explants. PCLS can serve as a bridge from animal models to human translation and can test many different pharmacological agents, although more studies are needed to fully validate the technology. Use of human PCLS also may help circumvent concerns about the differences in inflammatory or fibrogenic pathways between rodents and humans [52].

Clinical trials – state of the art and unmet needs

The accelerating progress in understanding mechanisms of hepatic fibrosis and defining therapeutic targets has heightened the urgency to establish clinical trial designs that can accurately assess efficacy of antifibrotic drugs. Before defining clinical trial features, however, it is important to understand the dynamics of fibrosis regression now that effective disease specific drugs have been developed for several liver diseases. Importantly, standard systems of fibrosis staging (e.g. Ishak, Brunt, Metavir) may not be as accurate as direct collagen proportionate area quantification, which correlates extremely well with clinical outcomes [53]. Regardless of the morphologic method, sustained suppression of hepatitis B yields remarkable improvements in inflammation and necrosis within one year, with subsequent improvement in fibrosis that is demonstrable at five years [29,54]. Similarly, effective treatment of hepatitis C also leads to remarkable fibrosis regression [30,55]. Although published experience is less extensive than for HBV, more information about fibrosis regression in chronic hepatitis C is expected now that highly effective direct acting antiviral drugs are entering widespread use. Remarkably, cirrhosis as defined by standard staging criteria is reversible in up to two thirds of patients when effectively treated with antiviral agents for HBV or HCV [29,30,54,55]. Emerging data from a trial treating NASH patients with bariatric surgery suggests remarkably similar patterns of improvement, with dramatic reductions in NASH activity score at one year but more modest antifibrotic effects during this interval [56]; longer term follow-up is still pending in these patients, but continued fibrosis regression is anticipated. In aggregate, these findings are important because they establish a realistic timeframe during which efficacy of an antifibrotic drug can be detected. Specifically, while significant improvements in necroinflammation often occur within one year, drugs targeting only fibrosis will likely take longer to establish their efficacy based on histologic criteria.

These findings also underscore the increasing limitations imposed by reliance on liver biopsy in clinical trials, not only

Table 1. Major studies with liver fibrosis as primary or co-primary endpoint. Modified from [27,68].

Cause	Drug name (action), treatment, patients included (F, C, NR, SVR)	Efficacy	Year of completion/ publication	Phase	No. of patients	NCT Ref.
HCV (not exclusively antiviral agents)	Farglitazar (PPARγ agonist); 52 wk, r, db (F/NR)	No effect	2010	2	225/265	[31]
	GS-9450 (pan-caspase inhibitor) *vs.* plac; 24 wk, nr, db (F/NR)	No results reported	2010	2	307	00874796
	Irbesartan (AT1R antagonist) *vs.* plac; 2 yr, r, db (F/NR)	Pending	2013	3	166	00265642
	Fuzheng Huayu (Chinese herbal drug) *vs.* plac, 48 wk, r, db (F)	Pending	2014	2	100	00854087
	Pirfenidone (anti-inflammatory) *vs.* plac, 2-yr intervention	Pending	2014	2-3	150	02161952
HBV (not exclusively antiviral agents)	Fuzheng Huayu *vs.* plac; 6 mo, r, db (F); biopsy and serum fibrosis markers	Significant for fibrosis regression and fibrosis markers	2005		226	[57]
	FG-3019 (anti-CTGF mAb) *vs.* entecavir *vs.* plac; 45 wk, r, db (F)	Pending	2016	2	228	01217632
	Entecavir ± Fuzheng Huayu *vs.* plac, 48 wk, r, db (C)	Pending	2016	4	700	02241590
HBV/HCV, coinfected	Oltipraz (Rock-kinase inhibitor) *vs.* plac; 24 wk, r, db (F,C)	No effect	2007/2011	2	83	00956098
PBC	UDCA *vs.* plac; 2 yr, db (F,C)	No effect	1991	3	146	[58]
	UDCA *vs.* plac; 4 yr, r, db (F,C)	Lower fibrosis progression	2000	4	103	[59]
	Obeticholic acid (FXR agonist) *vs.* plac; 12 mo-8 yr, r, db (F); UE and serum fibrosis markers	Pending	2023	3b	350	02308111
Alcoholic hepatitis	Candesartan (ACE inhibitor); 6 mo, r, db (F)	Significant histological improvement; 33.3% *vs.* 11.6%	2009/2012	1-2	85	[60]
PSC	GS-6624 (anti-LOXL2 mAb) *vs.* plac; 96 wk, r, db (F)	Pending	2015	2	225	01672853
NASH	Orlistat (pancreatic lipases inhibitor) *vs.* 1400 kcal diet (30% fat); 36 wk, r, ol (F)	No results reported	2006	4	50	00160407
	Pioglitazone (PPARγ agonist) *vs.* plac; 6 mo, r, db	No effect	2006	4	55	[61]
	Pioglitazone *vs.* plac; 1 yr, r, db (F)	Decreased fibrosis	2008	-	74	[62]
	Pioglitazone *vs.* Vit E *vs.* plac; 2 yr, r, db (F)	Improved inflammation, in all treatment arms trend for decreased fibrosis	2009-2010	3	247	[63]
	Rosiglitazone (PPARγ agonist) *vs.* plac; 1 and 2 yr, r (F)	No effect on fibrosis	2010	-	53	[64]
	Pentoxifylline (anti-TNFα) *vs.* plac; 1 yr, r, db (F)	Improved steatosis, inflammation and fibrosis	2010-2011	2	55	[65]
	Rosiglitazone (R) *vs.* R + metformin *vs.* R + losartan; 48 wk, r, ol (F)	No effect on fibrosis	2011	-	137	[66]
	High-dose UDCA *vs.* plac, 1 yr, r, db (F)	Significant reduction only of FibroTest	2011	3	126	[67]
	Metformin (AMP kinase agonist, anti-diabetic); 1 yr, r, db (F)	No results reported	2012	4	80	00134303
	Metformin *vs.* insulin; 1 yr, r, (C)	Pending	2016		126	02234440
	Liraglutide (GLP-1 agonist) *vs.* plac; 48 wk, r, db (F)	No results reported	2013	2	52	01237119
	Pentoxifylline + Vit E *vs.* Vit E; 3 mo (biopsy), r, db (F)	No results reported	2013	3	120	01384578
	Losartan (AT1R antagonist) *vs.* plac; 2 yr, r, db (F)`	Pending	2014	3	214	01051219
	Obeticholic acid (FXR agonist) *vs.* plac; 72 wk, r, db (F)	Significant for steatosis and inflammation; marginal effect on fibrosis	2014	2	280	[68]
	Pioglitazone (PPARγ agonist) *vs.* Vit E *vs.* plac; 1.5 and 3 yr, r, db (F)	Pending	2014	4	90	00994682
	GS-6624 (anti-LOXL2 mAb; 75 mg *vs.* 125 mg) *vs.* plac; 100 wk, r, db (F)	Pending	2015	2	225	01672866
	GS-6624 (200 mg *vs.* 700 mg) *vs.* plac; 100 wk, r, db (F,C)	Pending	2015	2	225	01672879
	GFT505 (dual PPAR α/δ agonist); 52 wk, r, db (F)	Pending	2015	2	270	01694849
	Pioglitazone (Pio) *vs.* Vit E *vs.* Vit E + Pio *vs.* plac; 1.5 and 3 yr, r, db (F)	Pending	2015	4	90	01002547
	Vi. D *vs.* lifestyle counseling; 2 yr, r, ol (F)	Pending	2014	3	200	01623024
	Vi. D3 *vs.* plac; 48 wk, r, db (F)	Pending	2015	2	60	01571063
	Omega-3 (fish oil) *vs.* plac; 1 yr, r, db (F)	No results reported	2010	2/3	64	00681408
	Omega-3 (fish oil); 18 mo, r, sb (F)	No results reported	2013	2	100	00760513
	Docosahexaenoic acid; 2 yr, r, db (F)	No results reported	2011	1/2	60	00885313
	Eicosapentaenoic acid *vs.* plac; 1 yr, r, db (F)	No results reported	2012	2	243	01154985
	Diamel (dietary supplement) *vs.* plac *vs.* lifestyle counseling; 52 wk, r, db (F)	No results reported	2012	3	158	00820651

ACE, angiotensin-converting enzyme; AT1R, angiotensin II receptor type 1; C, cirrhosis; CTGF, connective tissue growth factor; db, double-blind; F, fibrosis; IFN, interferon; FXR, farnesoid X receptor; GLP-1, glucagon-like peptide-1, IL, interleukin; LOXL2, lysyl oxidase-like 2; mAb, monoclonal antibody; NCT, number at ClinicalTrials.gov; NR, non-responders; nr, non-randomized; ol, open-label; r, randomized; retro, retrospective analysis; TNFα, tumor necrosis factor α; UDCA, ursodeoxycholic acid; UE, ultrasound elastography; Vit, vitamin.

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because of its invasive nature and its propensity to sampling variability, but also because there may be earlier changes in fibrogenic activity that may occur earlier than could be detected by biopsy. To date, however, no noninvasive markers can reliably detect evidence of an antifibrotic effect, although clinical trials are already incorporating such exploratory markers as secondary endpoints.

These obstacles notwithstanding, a remarkable number of clinical studies with liver fibrosis as primary or co-primary endpoint have been initiated and even concluded. Table 1 highlights the results of some of these clinical trials [57–68].

Markers of fibrosis and fibrogenic activity

A broad range of noninvasive markers is under intensive study to complement or replace biopsy in future trials (Table 2). These include vibration controlled shear wave elastography [69], magnetic resonance elastography [70], acoustic force radiation impulse force impulse imaging [71], magnetic resonance techniques to determine the inflammation and fibrosis score [72] or that specifically find and quantify ECM molecules [73], dynamic markers of collagen synthesis using non-radiolabeled isotopes [74], PET imaging to label fibrosis-specific cells or receptors [27], as well as a growing list of functional studies. Such functional studies may not directly reflect histology, but could be far more sensitive in determining liver reserve and clinical outcomes, much as spirometry is a vital endpoint in trials of antifibrotic drugs for idiopathic pulmonary fibrosis. Functional studies currently under evaluation include a cholate clearance test which has been extensively validated in HCV patients [75] as well as a breath test that assesses the ability of microsomal enzymes to metabolize a ^{13}C labeled orally administered substrate to release $^{13}CO_2$ in the nasal breath [76].

In addition to general tests of liver integrity or function, there may additionally be disease- or drug-specific tests to establish proof of target engagement or mechanism. For example, antagonism of a specific signaling pathway could include patients with more advanced disease and might rely upon a biomarker that specifically reflects blockage of the target pathway either in tissue, by imaging or in blood.

Table 2. Potential endpoints for clinical trials.

1.	**Liver histology**
a.	Necroinflammation
i.	NAFLD Activity Score
ii.	Knodell score
b.	Fibrosis
i.	Fibrosis staging-Brunt, Metavir, Ishak
ii.	Collagen proportionate area
c.	Markers of fibrogenic cell activity
i.	Alpha smooth muscle actin quantification
ii.	Beta PDGF receptor quantification
2.	**Liver stiffness**
a.	Vibration controlled transient elastography
b.	Shear wave elastography
c.	Acoustic radiation force impulse imaging
d.	Magnetic resonance elastography
3.	**MR or PET-based technologies**
a.	Liver inflammation score
b.	Proton density fat fraction
c.	Collagen or lysyl oxidase content using specific contrast agent
d.	Receptor binding by PET ligands
4.	**Serum tests**
a.	Fibrogenic panels that include ECM molecules
b.	Other serum marker panels
i.	FIB-4
ii.	ELF test
iii.	APRI
c.	Lipidomic profiles or markers
5.	**Functional tests**
a.	Cholate clearance
b.	^{13}C methacetin breath test
c.	Indocyanine green clearance tests
d.	Galactose elimination tests
e.	Collagen synthesis quantification measuring ^{13}C labeled turnover
6.	**Clinical Scores**
a.	MELD score
b.	Child-Pugh score
c.	Maddrey discriminant function (for alcoholic liver disease)
d.	Lille Score (for alcoholic liver disease)
e.	Combined clinical/pathologic scores (for alcoholic liver disease)

Current concepts of clinical trial design – a rapidly moving target

Despite the limitations of current noninvasive markers, the urgent need to develop new antifibrotic drugs, combined with the impressive success of candidate therapies in animal models demands that trials be initiated without delay while we accrue further information about noninvasive markers. The rapidly changing landscape of clinical trial design for liver disease is recognized by regulatory agencies in the United States (FDA) and Western Europe (EMA), who are working together with the broad range of stakeholders to standardize approaches to clinical trial design. Similar collaborative models of stakeholder engagement have been successfully employed to accelerate progress in clinical trial design for HIV and HCV [77], and thus optimism prevails that similar cooperation in standardizing approaches to antifibrotic therapies will accelerate progress. Moreover, a series of consensus conferences and meetings of key opinion leaders is also contributing to the rapid consolidation of knowledge and accelerated development of new trial strategies.

At present, NASH is the dominant disease indication for candidate antifibrotic drugs. This focus on NASH reflects both the astonishing improvement in the specific therapies for viral hepatitis, combined with the growing appreciation of the magnitude of the obesity and fatty liver disease epidemics, which affects up to tenfold more individuals than HCV in the US and Europe. Moreover, specific ethnicities are at heightened risk of fatty liver disease at relatively lower BMIs, including Latin Americans, and Asians.

Fig. 3 summarizes potential variables for patient stratification in antifibrotic clinical trials. The first criterion for clinical trial design is to clearly define patients who are at the greatest risk for disease progression, and to ensure that these risk factors are equally distributed between placebo and control groups in randomized controlled trials. While genetic factors may contribute up to 20% of the risk in NASH, at present these genetic factors cannot be reliably used to stratify risk. Instead, the presence of diabetes, older age, elevated ALT, and severe BMI elevation are

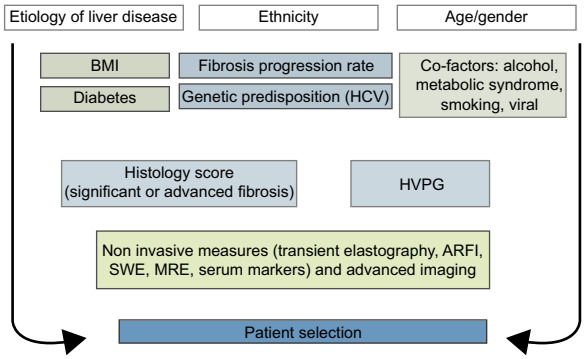

Fig. 3. Potential variables for patient stratification in antifibrotic clinical trials. ARFI, Acoustic radiation force impulse imaging; BMI, body mass index; HVPG, hepatic vein pressure gradient; MRE, magnetic resonance elastography, SWE, shear wave elastography.

more prevalent risk factors for progression [78] and must be distributed equally among study groups in a clinical trial.

An additional element to be used in stratifying patients is the stage of disease. Drugs that target inflammation and cell injury are likely to be effective at earlier and intermediate stages of the disease, and thus trials testing these agents might avoid patients with more advanced fibrotic stages to establish proof of principle in phase 2 trials. In contrast, drugs targeting mechanisms that are more important as disease advances could favor the enrollment of more advanced patients, which in turn allows the inclusion of endpoints that reflect more advanced disease stages. These endpoints could include hepatic venous pressure gradient [79], which correlates strongly with clinical outcomes, as well as MELD or Child-Pugh scores.

Ultimately, approval of antifibrotic drugs will rely on endpoints that have either a direct correlation with clinical outcomes, or are reasonably likely to predict clinical outcomes, since effective therapies must improve how a patient "feels, functions, or survives", according to FDA. Thus, endpoints such as hepatic vein pressure gradient, which reliably correlate with clinical outcomes, have a special appeal for inclusion in antifibrotic drug trials.

Following clarification of enrollment criteria and patient selection, the next challenge in trial design is whether biomarkers will be incorporated for interval analysis of efficacy. As noted, there are no validated noninvasive markers to indicate a drug is showing efficacy at intermediate time points, so that current trial designs are likely to rely upon liver biopsy, and would need to be conducted for at least one year, and preferably longer. Nonetheless, incorporation of exploratory biomarker assessments such as imaging and functional tests in the current generation of clinical trials could lead to their inclusion as primary endpoints in later trials.

Trial design for liver diseases other than NASH is even more problematic. In principle, alcoholic liver disease could rely not only on biopsy, but also recent disease specific scoring systems that include clinical and pathologic data [80]. Drug therapy of this disease is especially challenging because of the confounding effects of continued alcohol use or its cessation in the midst of a trial. Even more vexing is primary sclerosing cholangitis, in which biomarkers of disease and risk factors for progression are poorly understood. In fact, imaging tests in this disease do not reliably reflect fibrosis, and large duct disease cannot be assessed by liver biopsy. Nonetheless, the severity of this disease, its high propensity to develop cholangiocarcinoma, and the absolute absence of therapies demand intensive efforts to identify disease specific biomarkers that can be used as endpoints in clinical trials of anti-inflammatory or antifibrotic drugs for this condition.

In summary, now that there is intense focus on development and testing of drugs to treat hepatic fibrosis, consolidation and progress in developing endpoints and clinical trial designs is imminent. It is anticipated that guidelines in this review will rapidly become outdated as the field extends into prospects for attenuating CLD that could not have been imagined 30 years ago when the *Journal of Hepatology* was launched.

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Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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