Hepatic Fibrosis

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Biomarkers of hepatic fibrosis, fibrogenesis and genetic pre-disposition pending between fiction and reality

O. A. Gressner, R. Weiskirchen, A. M. Gressner *

Institute of Clinical Chemistry and Pathobiochemistry, Central Laboratory, RWTH-University Hospital, Aachen, Germany

Abstract

Fibrosis is a frequent, life-threatening complication of most chronic liver diseases. Despite major achievements in the understanding of its pathogenesis, the translation of this knowledge into clinical practice is still limited. In particular, non-invasive and reliable (serum-) biomarkers indicating the activity of fibrogenesis are scarce. Class I biomarkers are defined as serum components having a direct relation to the mechanism of fibrogenesis, either as secreted matrix-related components of activated hepatic stellate cells and fibroblasts or as mediators of extracellular matrix (ECM) synthesis or turnover. They reflect primarily the activity of the fibrogenic process. Many of them, however, proved to be disappointing with regard to sensitivity and specificity. Up to now hyaluronan turned out to be the relative best type I serum marker. Class II biomarkers comprise in general rather simple standard laboratory tests, which are grouped into panels. They fulfil most criteria for detection and staging of fibrosis and to a lesser extent grading of fibrogenic activity. More than 20 scores are currently available, among which Fibrotest™ is the most popular one. However, the diagnostic use of many of these scores is still limited and standardization of the assays is only partially realized. Combining of panel markers in sequential algorithms might increase their diagnostic validity. The translation of genetic pre-disposition biomarkers into clinical practice has not yet started, but some polymorphisms indicate a link to progression and outcome of fibrogenesis. Parallel to serum markers non-invasive physical techniques, for example, transient elastography, are developed, which can be combined with serum tests and profiling of serum proteins and glycans.

Keywords: liver fibrosis ● liver fibrogenesis ● biomarkers ● serum markers ● genetic biomarkers ● fibrosis scores ● hepatic stellate cells ● TGF-β

Introduction

The ultimate goals of biomedical research are focused on the translation of new pathogenetic insights to clinical practice. As examples improved diagnosis and follow-up, more efficient and specific

*Correspondence to: Olav A. GRESSNER
RWTH University Hospital, Institute or Clinical Chemistry
and Pathobiochemistry–Central Laboratory,*

Pauwelsstr. 30, 52074 Aachen, Germany
Tel.: 00 49-(0)24 1-80-88 67 1; Fax: 00 49-(0)24 1-80-82 51 2
E-mail: agressner@ukaachen.de

therapeutic modalities, and identification of (genetic) risk factors or precipitating mechanisms for a given clinical condition are important clinical requirements and, thus, challenges for translational research. These efforts are clearly visible in the current research on cellular and molecular mechanisms of fibrosis in the liver and other organ fibrosis as well, which has brought over the last 20 years or so a plenitude of new insights into (*i*) the composition of the fibrotic extracellular matrix (ECM), (*ii*) cellular sources of ECM, (*iii*) the nature of the molecular mediators regulating ECM expression, ECM turnover and paracrine cellular interactions, (*iv*) resolution of ECM, apoptosis of contributing cells (hepatic stellate cells [HSC], hepatocytes) and reversibility of fibrosis. Goals are to find therapeutic options, at least experimentally, and to establish innovative non-invasive biomarkers indicating the activity (progression) of development (fibrogenesis) rather than the stage (extent) of fibrotic organ transition [1, 2].

However, up to now, actual clinical handling of liver fibrotic patients did not profit so much from biomedical fibrosis research. Ongoing clinical trials, however, are promising that therapeutic breakthroughs and improvements of diagnosis can be expected in the near future [3].

Therapeutic trials need frequent, reliable, objective and cost-effective diagnostic and follow-up procedures, which complement liver biopsies as 'surrogate markers'. Besides invasiveness (mortality rate $1:10^3$ to $1:10^4$, severe complications in 0.57% of cases) and the likelihood of sampling error [$1/50\,000^{th}$ (about 30 mg) of the liver mass (~ 1500 g) can hardly be representative for the whole organ] of biopsy histological examination depends on sample quality, that is, on length and size of the tissue specimen (co-efficient of variation between 45 and 55%, accuracy 65–75%) [4] and on the subjective evaluation of morphological changes ('observer error') including grading of necro-inflammatory activity (the driving force of fibrogenesis) and staging (extent) of fibrotic organ transition [5, 6]. Thus, the diagnostic value of the biopsy as 'gold standard' in the detection of fibrosis/fibrogenesis must be questioned. This situation strengthens the need for harmless, alternative or complementary serum biomarkers. Since they derive in part from pathogenetic pathways, a brief overview on fibrogenesis facilitates their understanding.

Pathways, cells and molecular mediators in liver fibrogenesis

Current knowledge ascribes liver-specific pericytes, that is, HSC, a major role in ECM production and re-modelling [7–10]. HSC, formerly termed vitamin A-storing cells, fat-storing cells, lipocytes or Ito-cells are located in the sub-endothelial space of Disse in close proximity to hepatocytes embracing with star-like extensions (spines) the sinusoidal endothelial tube [11]. They express some heterogeneity and represent about 15% of total resident liver cells and about 30% of non-parenchymal cells including Kupffer cells, sinusoidal endothelial cells and pit cells [12]. The ultra-structural features are characterized by large triacylglycerol-filled vacuoles containing retinoids [11, 13]. Besides their function as major vitamin A storage sites [14], that is, around 85% of liver vitamin A is located in this cell type, HSC were recently identified as antigen-presenting cells (APC) [15, 16] and are likely to have additional functions in liver cell renewal, regeneration, immunoregulation, angiogenesis and vascular re-modelling [17]. Their dominant role in fibrogenesis is based on their ability to change the phenotype from retinoid-storing, resting cells to contractile, smooth-muscle α-actin positive, vitamin A-depleted myofibroblasts (MFB) with a strongly developed endoplasmic reticulum and Golgi-apparatus if HSC are challenged by necro-inflammatory stimuli [1, 18]. Myofibroblasts synthesize and secrete virtually all of the matrix components found in ECM of the fibrotic liver (Fig. 1). This, however, does not rule out the contribution of other cell types and mechanisms to enhance matrix production in chronically inflamed liver tissue. The role of portal (MFB), in particular in biliary fibrosis, has been emphasized [19, 20] and, recently, the influx on bone-marrow-derived fibrocytes [21] *via* the circulation into the damaged tissue has been shown [22–24] Similarly, circulating monocytes, monocyte-like and mesenchymal stem cells have the potential to change to fibroblasts and other cell types if the appropriate microenvironment is provided [25]. Furthermore, actual research is focused on the possibility of epithelial-mesenchymal transition (EMT) [26], which describes the transition of biliary epithelial cells or even of hepatocytes to fibroblasts, which participate actively in the generation of fibrotic ECM.
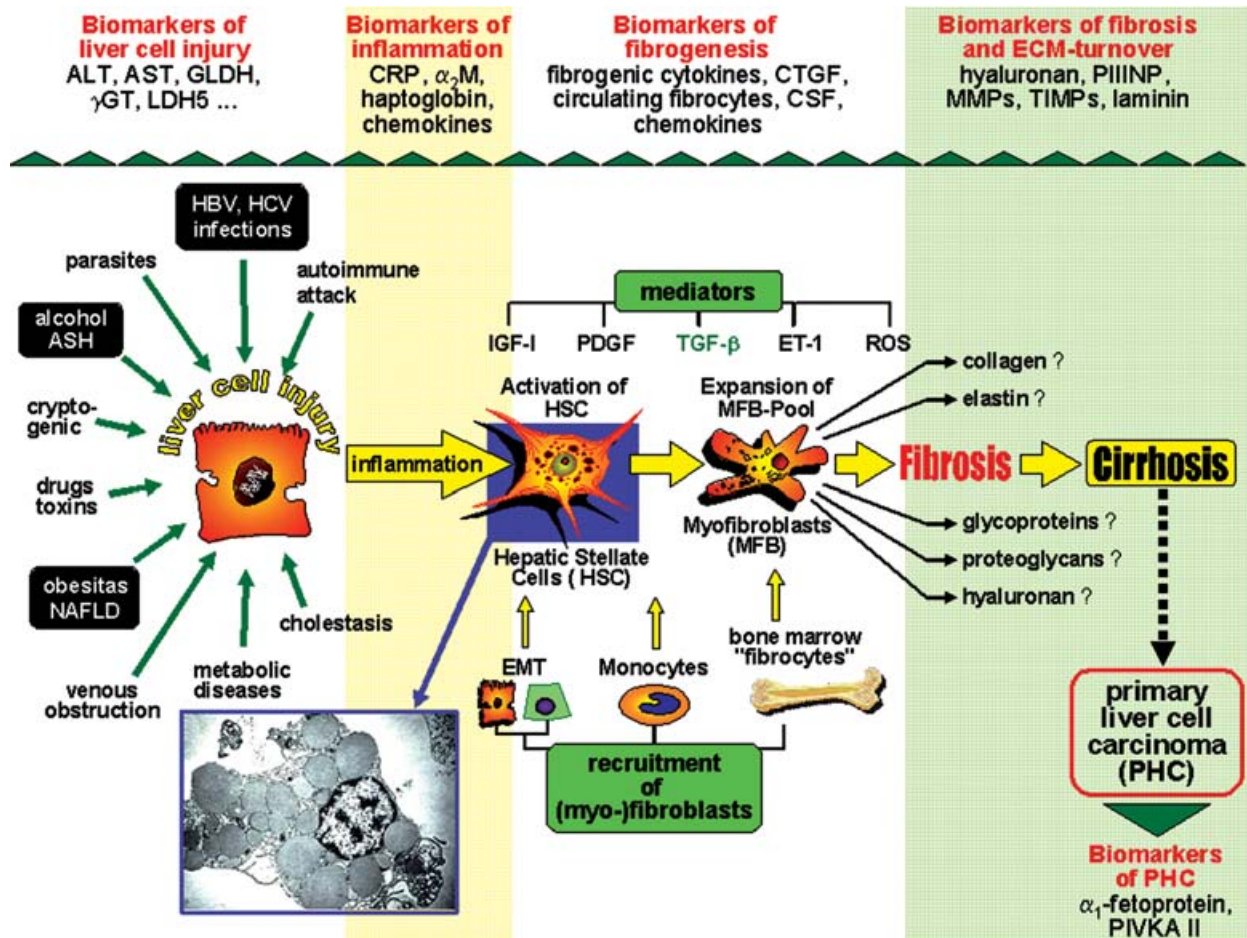
Fig. 1 Schematic presentation of the pathogenetic sequence of liver fibrosis and cirrhosis based on the activation of hepatic stellate cells (HSC) and transdifferentiation to matrix-synthesizing myofibroblasts (MFB). The inset of the electron micrograph shows retinoid-filled lipid droplets of HSC indenting the nucleus. Surrogate pathogenetic mechanisms contributing to the expansion of the myofibroblast pool in fibrotic liver are indicated: epithelial-mesenchymal-transition (EMT) of biliary epithelial cells or even hepatocytes, transformation of circulating monocytes at the site of injury to fibroblasts and the influx of bone marrow-derived fibrocytes into damaged tissue. Examples of serum biomarkers reflecting the pathogenetic sequence are given, but a considerable overlap is noticeable. Abbreviations: see Table 2, CRP, C-reactive protein; CSF, colony-stimulating factor; CTGF, connective tissue growth factor; GLDH, glutamate-dehydrogenase; PIVKA, prothrombin induced by vitamin K absence

However, the role of EMT in liver fibrogenesis is still under debate, but is well established in lung and kidney fibrosis [26].

The molecular mediators of the complex cellular network between stellate cells, resident liver cells, platelets and invaded inflammatory cells are mostly known (Fig. 2). The fibrogenic master cytokine is transforming growth factor (TGF)-β [10, 27] followed by platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), endothelin-1, angiotensin II

and certain fibroblast growth factors, but also non-peptide signalling components, such as acetaldehyde (in alcoholic fibrosis) and reactive oxygen species and H_2O_2 are noteworthy [11]. The bioactive, 25 kD TGF-β homodimer not only activates HSC, but stimulates ECM synthesis in HSC/MFB and fibroblasts/fibrocytes. Furthermore, TGF-β is a driving cytokine of EMT, stimulates chemokine (receptor) expression, apoptosis of hepatocytes (a pre-requisite for fibrogenesis) and decreases ECM catabolism by
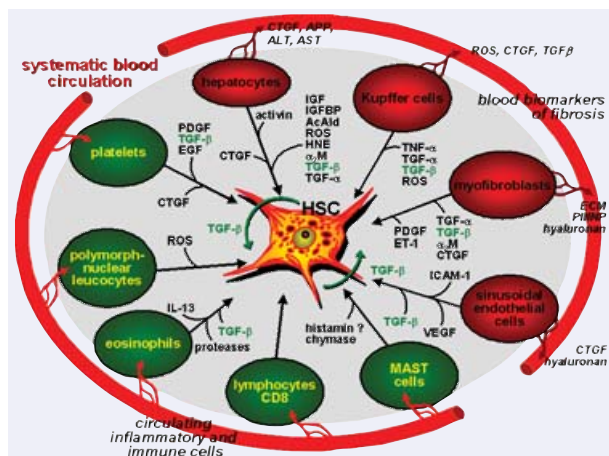
Fig. 2 Network of resident liver cells (red) and inflammatory non-liver resident cells (black) with hepatic stellate cells in the process of activation and transdifferentiation to myofibroblasts. Major molecular mediators are indicated. The influx of inflammatory and immune competent cells from the circulation into the damaged liver tissue is illustrated. Secreted products of resident liver cells leading to biochemical changes in blood of liver fibrotic patients are exemplified. Abbreviations: AcAld, acetaldehyde; $\alpha_2 M$, α_2-macroglobulin; CTGF, connective tissue growth factor; EGF, epidermal growth factor; ET-1, endothelin-1; HNE, 4-hydroxynonenal; HSC, hepatic stellate cells; ICAM-1, intercellular adhesion molecule-1; IGFBP, IGF-binding proteins; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

down-regulation of matrix metallo-proteinases (MMPs) and up-regulation of tissue inhibitor of metalloproteinase (TIMPs), the specific tissue inhibitors of MMPs [28]. Several other functions of TGF-β are known including a strong immunosuppressive effect, mitogenic or anti-proliferative actions (depending on the cell type), regulation of cell differentiation and tumour suppression in the early stage. Thus, there is a need to regulate the activity of TGF-β sensitively by extracellular proteolytic activation of a large molecular weight precursor (large latent TGF-β complex). The latent TGF-β complex is the primary secretion product of TGF-β, which can be covalently fixed in the fibrotic ECM by a transglutaminase-dependent reaction. Bioactive TGF-β is released by proteolytic truncation of the complex. Furthermore, bone morphogenetic protein-7 (BMP-7), a member of the TGF-β gene superfamily, is a potent antagonist of TGF-β, for example, an inhibitor of TGF-β-driven EMT and apoptosis [26, 29]. BMP-7 reverses TGF-β signalling, which occurs

via phosphorylated Smad proteins transferring the signal from the serine-threonine-kinase receptors to the Smad-binding elements in the promoter region of TGF-β target genes. One of these TGF-β-dependent genes is that of connective tissue growth factor (CTGF/CCN2), a cysteine-rich, secreted, 38 kD multi-domain protein, which has an important role as a downstream modulator of TGF-β effects [30, 31]. CTGF synthesis is not limited to HSC and (MFB). Instead, TGF-β-dependent CTGF gene expression and secretion was recently shown to occur in hepatocytes in culture and in experimental liver fibrosis [32]. Additional antagonists of TGF-β are synthetic and naturally occurring PPAR-γ agonists like prostaglandin J2 (PGJ2), thiazolidone and triterpenoids [33]. These chemicals might gain therapeutic application in human fibrosis. Due to its multiple functions TGF-β is termed 'plasticity-factor', notifying its extensive cross-talk with other cytokines and signalling pathways, for example, p38 MAP kinases, ERK and JNK.

Classification of biomarkers of fibrosis

Serum- or plasma-based biomarkers of liver fibrogenesis/fibrosis can be sub-divided in two classes: Class I fibrosis biomarkers are pathophysiologically derived from ECM turnover and/or from changes of the fibrogenic cell types in liver described above. They should reflect the activity of the fibrogenic and/or fibrolytic process and, thus, re-modelling of ECM. These biomarkers do not indicate the extent of connective tissue deposition, that is, the stage of fibrotic transition of the organ. Frequently, they are costly laboratory tests and are the result of translation of fibrogenic mechanisms into clinical application. Thus, their selection is hypothesis-driven. In contrast, class II fibrosis markers have been statistically proven (multi-variate analyses) to be best correlated with fibrosis and to a lesser extent with fibrogenesis or fibrolysis. Class II markers mostly estimate the degree of fibrosis (extent of ECM deposition). In general, they comprise common clinical-chemical tests (enzymes, proteins, coagulation factors), which do not necessarily reflect ECM metabolism or fibrogenic cell changes. Their pathobiochemical connection with fibrogenesis is indirect if at all. Thus, their selection is not hypothesis-driven, but empiric. The

Table 1 Class I biomarkers of liver fibrogenesis

Extracellular matrix-related enzymes			
Enzyme	**Specimen**		**Method**
	Serum	Urine	biopsy Liver	
Prolylhydroxylase	+	-	+	Radio-enzymatic, RIA
Monoamine-oxidase	+	-	(+)	Enzymatic
Lysyloxidase	+	-	+	RIA
Lysylhydroxylase	+	-	-	RIA
Galactosylhydroxylysyl-glucosyltransferase	+	-	+	RIA
Collagenpeptidase	+	-	+	Enzymatic
N-Acetyl-β-D-glucosaminidase	+	+	+	Enzymatic

Collagen fragments and split products			
Type of collagen	**Specimen**		**Method**
	Serum	Urine	Liverbiopsy	
Type I pro-collagen				
• N-terminal pro-peptide (PINP)	+	-	+	ELISA
• C-terminal pro-peptide (PICP)	+	-	+	RIA
Type III pro-collagen				
• Intact pro-collagen	+	-	-	RIA
• N-terminal pro-petide (PIIINP)				
• Complete pro-peptide (Col 1-3)	+	-	-	RIA
• Globular domain of pro-peptide (Col-1)	+	-	-	RIA
Type IV collagen				
• NC1-fragment (C-terminal cross-linking domain [PIVP])	+	+	-	ELISA, RIA
• 7S domain ('7S Collagen')	+	+	-	RIA
Type VI-Collagen	+	+	+	RIA

Glycoproteins and matrix-metalloproteinase (inhibitors)			
Marker	**Specimen**		**Method**
	Serum	Urine	Liver tissue	
Laminin, P1-fragment	+	-	-	RIA, EIA
Undulin	+	-	-	EIA
Vitronectin	+	-	-	EIA
Tenascin	+	-	-	ELISA
YKL-40	+	-	+	RIA/ELISA
(Pro)matrix metalloproteinase (MMP-2)	+	-	-	ELISA
Tissue inhibitor of metalloproteinases (TIMP-1, TIMP-2)	+	-	-	ELISA
sICAM-1 (soluble intercellular adhesion molecule, sCD54) sVCAM-1 (soluble vascular cell adhesion molecule, sCD106)	+	-	-	ELISA

Table 1 Continued

Glycosaminoglycans				
Marker	**Specimen**			**Method**
	Serum	Urine	Liver tissue	
Hyaluronic acid (Hyaluronan)	+	-	-	Radioligand assay ELISA

Molecular mediators				
Marker	**Specimen**			**Method**
	Serum	Urine	Liver tissue	
Transforming growth factor β (TGF-β)	+	-	+	ELISA
Connective tissue growth factor (CTGF/CCN2)	+	?	+	ELISA

markers are standard laboratory tests and are integrated into multi-parametric panels.

In general, both types of serum biomarkers follow different pathophysiological concepts with diverse clinical implications. Class I markers inform about 'what is going on' (grade of fibrogenic activity), class II markers indicate 'where fibrosis is' (stage of fibrosis).

Class I biomarkers of fibrosis

This group of fibrogenic biomarkers comprises mainly secretion products of activated HSC and portal (MFB), *i.e.*, matrix components, ECM-related enzymes, TGF-β-dependent export proteins of hepatocytes and mediators of ECM-synthesis or turnover (Table 1). The elevation of these components in the circulation is due to increased expression of ECM-components in the fibrotic tissue (*e.g.* by HSC) and fractional spillover into the systemic bloodstream. Additionally, reduced clearance by Kupffer cells, sinusoidal endothelial cells or hepatocytes, for example, by perihepatic blood shunting or decrease of scavenger receptor functions of the respective cell types contributes to their elevation in blood (Fig. 3). Some of the previously recommended enzymes of collagen metabolism (*e.g.* prolylhydroxylase, lysyloxidase, -hydroxylase, collagen peptidase) have nowadays only anecdotic character because their activities in serum do not reflect reliably matrix synthesis, but cell necrosis (Table 1). In addition, their measurement is laborious and costly involving radio-enzymatic, mostly not standardized, cumbersome assays [34]. Similarly, catabolic enzymes of the glycoprotein and proteoglycan metabolism, such as β-glucuronidase and

N-acetyl-β-D-glucosaminidase have not convinced as biomarkers of liver fibrosis. Summarizing the plenitude of existing literature, only a few class I fibrosis biomarkers have reached a certain clinical importance [35–37]. In several studies, hyaluronan (formerly termed hyaluronic acid) currently proves to be the relative best class I biomarker of fibrosis having sensitivities and specificities of 86–100% and 88%, respectively, if cirrhosis in non-alcoholic fatty liver disease (NAFLD) [38] and other etiologies are considered [39]. The diagnostic power of hyaluronan is based on the high negative predictive value (98–100%) at a cut-off concentration of 60 µg/l, which is significantly higher than the positive predictive value of 61%. Promising diagnostic sensitivity and negative predictive value can be ascribed to the stimulated synthesis of hyaluronan in activated HSC [40], its direct secretion into the sinusoidal bloodstream, and the short half-lifetime of 2–9 min in the circulation, which is prolonged in disease conditions by a reduced clearance in the sinusoidal endothelial cells [41]. Of the several pro-collagen and collagen fragments proposed as biomarkers [35] only the aminoterminal propeptide of type III pro-collagen (PIIINP) has reached a limited, but no widespread and continuous clinical application [42]. Reported sensitivities and specificities vary considerably around 76–78% and 71–81%, respectively, which can be increased if combined with additional collagen fragment markers. It should be noticed that PIIINP, hyaluronan and several other class I fibrosis markers are not disease-specific, because elevations are also reported for rheumatoid diseases, chronic pancreatitis, lung fibrosis, scleroderma and others. A series of other studies was focused on the clinical
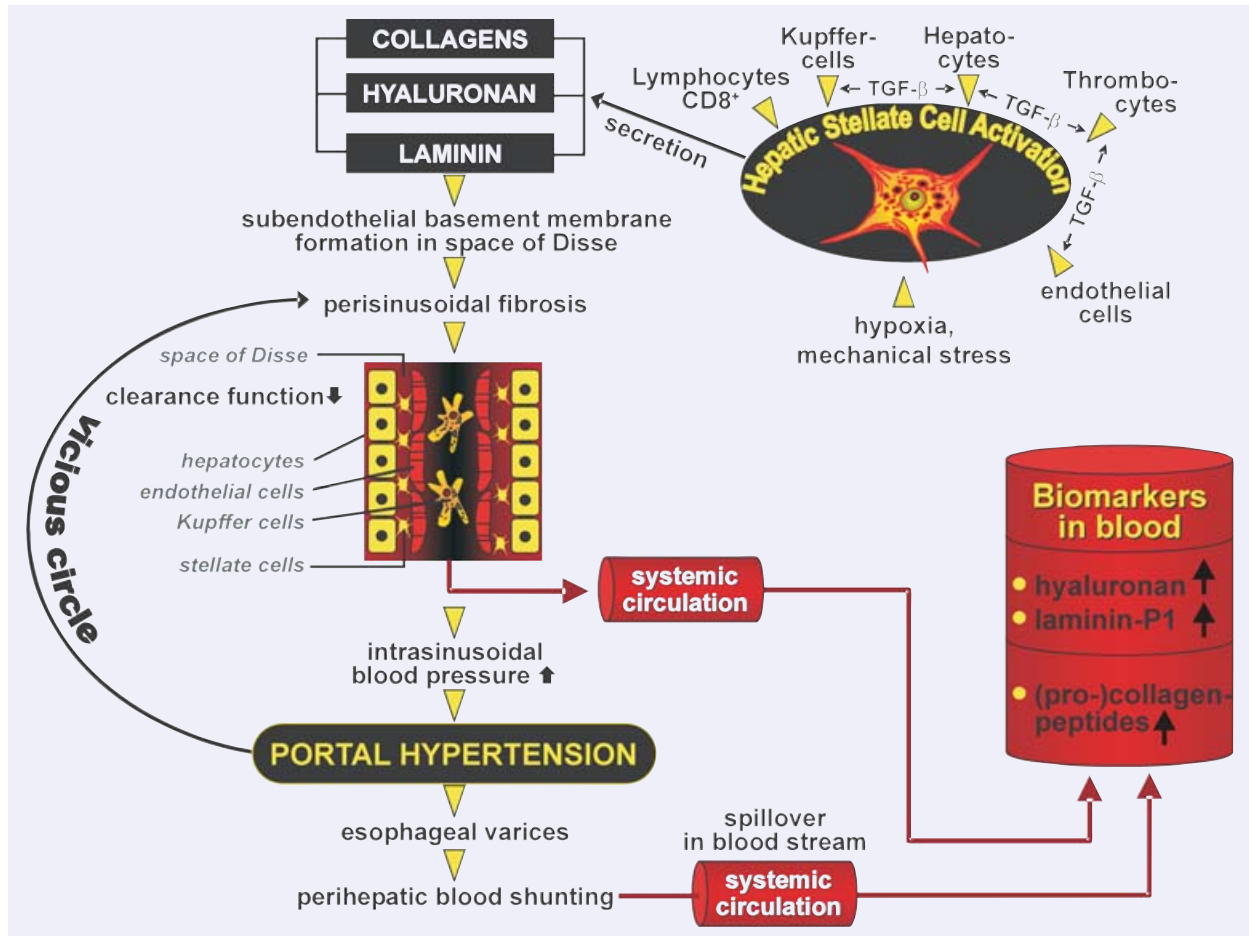
Fig. 3 Pathobiochemical mechanisms of elevation of class I biomarkers of fibrosis exemplified by collagens hyaluronan and laminin, respectively. Increased production by activated hepatic stellate cells due to paracrine stimulation via TGF-β by interacting cells, mechanical stress and hypoxia leads to stimulated secretion and consecutive deposition as incomplete basement membranes in the space of Disse and perisinusoidal fibrosis. As a consequence of newly developed subendothelial basement membrane and cellular insufficiency, the clearance function of the sinusoidal compartment for circulating matrix components is decreased and intrahepatic hemodynamic resistance is elevated. The latter leads to perihepatic shunting of blood reducing further the elimination of matrix components and their fragments from the blood.

significance of the elevated P1-fragment of the large molecular weight basement membrane glycoprotein laminin [43]. It was reported to be a predictor of portal hypertension, because a positive correlation was noticed between the portal venous pressure and the increase of the P1-laminin fragment in blood [44]. Positive and negative predictive values are given with 0.77 and 0.85, respectively, sensitivity and specificity with 87% and 74%, respectively [44]. If combined with hyaluronan [45] or aminoterminal pro-peptide of type III pro-collagen [46], the diagnostic criteria for assessing portal hypertension can be further improved.

As outlined above, TGF-β is clearly identified as a pro-fibrogenic master cytokine having a superior position in the hierarchy of fibrogenesis-stimulating growth factors. As a result, TGF-β concentrations in plasma were analysed to estimate their diagnostic significance. The concentrations are elevated in and correlated with the severity of liver diseases suggesting this cytokine as a non-invasive biomarker of hepatic dysfunction in chronic liver diseases [47], and possibly of hepatic fibrosis progression [48]. The significant correlation with aspartate-aminotransferase (AST) and alanin-aminotransferase (ALT)
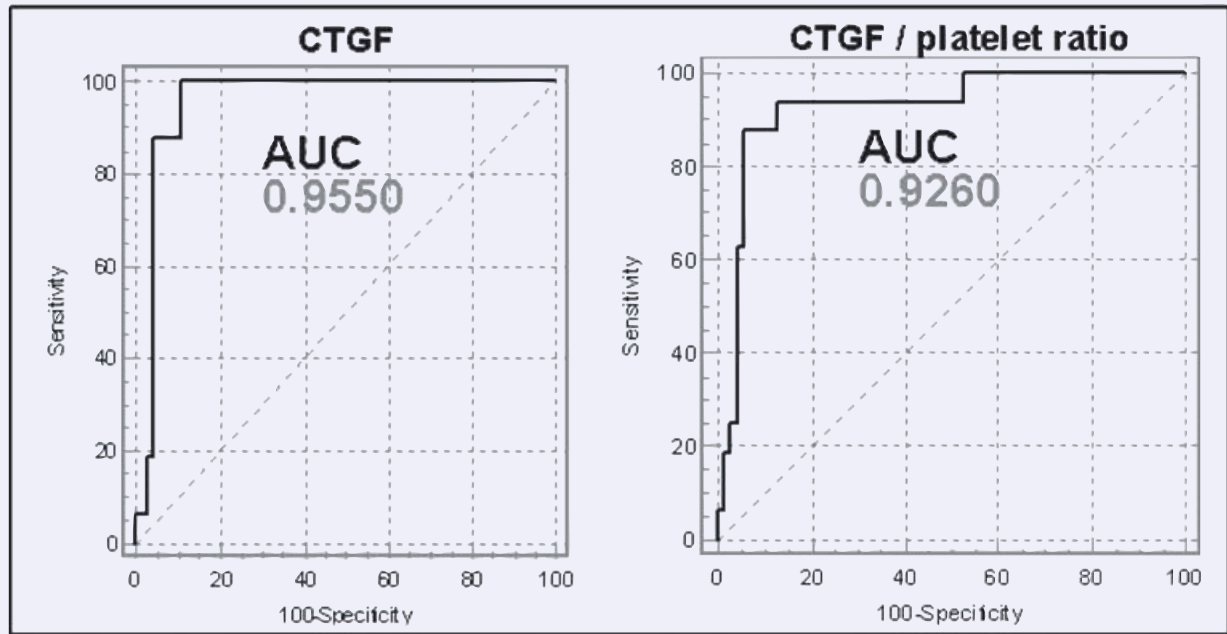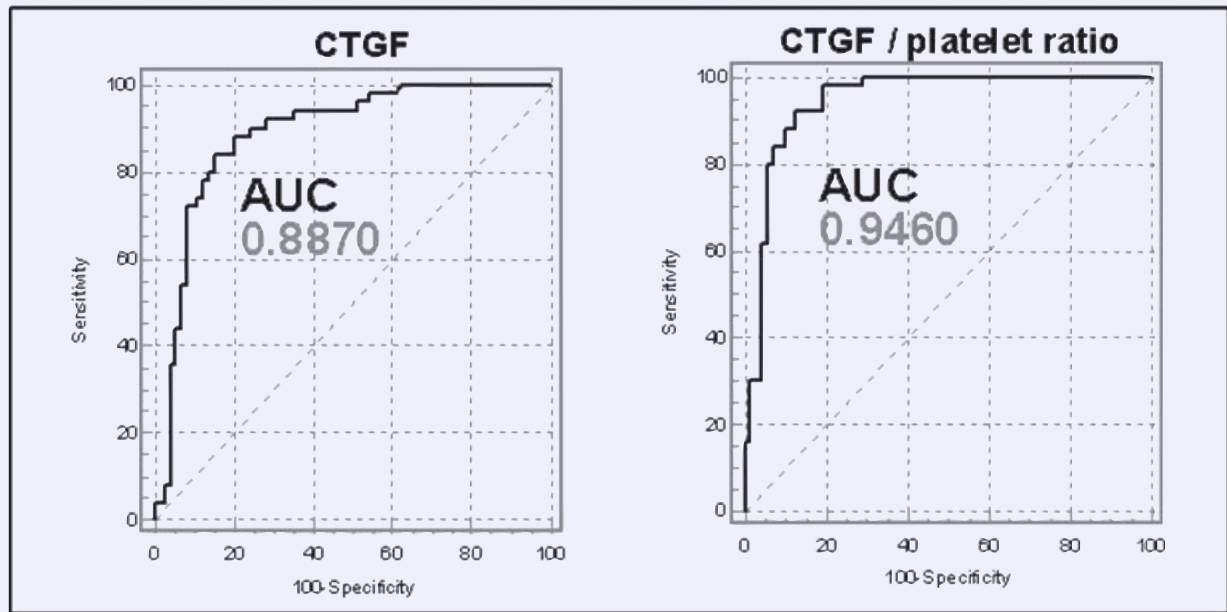
Fig. 4 Receiver-operating-characteristic (ROC) curves of the diagnostic power of serum CTGF and of the CTGF/platelet ratio for fibrosis and cirrhosis, respectively. AUC, area under the curve. Data compiled from ref. [55].

activity in serum [49] and the pathobiochemical finding that hepatocytes contain substantial amounts of TGF-β, which is released into the medium if hepato-cytes are damaged [50], proposes that the elevation of this cytokine in serum is due to necrosis instead of fibrogenesis. Since necrosis and consecutive

necro-inflammation are the driving forces of fibrogenesis, the elevation of TGF-β in serum/plasma might be an indirect clinical parameter of fibrogenesis. Additionally, decreased clearance of plasma TGF-α by impaired function of hepatocytes will contribute to its elevation in liver diseased patients [51], because parenchymal liver cells play a major role in uptake and clearance of this cytokine[52]. Furthermore, most of the circulating TGF-β is in a latent, biologically non-active status bound to carrier proteins, for example, α2-macroglobulin [53]. Thus, measurement of TGF-β requires transient acidification before total (active and latent) TGF-β can be quantified [54].

Preliminary studies point to CTGF/CCN2 in serum as an innovative class I biomarker of fibrogenesis [55]. This 38 kD protein is synthesized not only in HSC, but also in hepatocytes where the expression and secretion is strongly dependent on TGF-β [32]. Accordingly, CTGF expression in fibrotic liver tissue is up-regulated and its concentration in serum or plasma elevated if fibrogenesis is going on. There is a correlation between CTGF levels and fibrogenesis, because the levels decrease in fully developed, end-stage cirrhosis, compared to fibrosis. The area under the curve (AUCs) for fibrosis *versus* control and cirrhosis versus control were calculated to be 0.955 and 0.887, respectively, the sensitivities 100% and 84%, respectively, the specificities 89% and 85%, respectively (Fig. 4) [55]. These criteria suggest CTGF as a potentially valuable class I biomarker of active fibrogenesis.

Recently, the glycoprotein YKL-40 ('chondrex', molecular mass 40 kD), likely a growth factor for fibroblasts and endothelial cells, was shown to be strongly expressed in human liver tissue. In particular, HSC contain YKL-40 mRNA. Several studies have found elevated YKL-40 concentrations in sera of patients with liver diseases independent of disease etiology. Sensitivities and specificities around 80% and an AUC of 0.81 for fibrosis are reported for hepatitis C virus (HCV)-patients [56], for those with alcoholic liver disease, specificity of 88% and a low sensitivity of 51% were calculated [57]. Serum concentrations of this protein correlated with other ECM products secreted by HSC and fibroblasts, for example, PIIINP, hyaluronan, MMP-2 and TIMP-1. It is claimed that YKL-40 concentrations reflect the degree of liver fibrosis, but extensive clinical evaluation is required and other inflammatory diseases as potential conditions of YKL-40 elevations have to be excluded.

Class II fibrosis biomarkers

This category comprises a rapidly increasing, great variety of biochemical scores and multi-parameter combinations (biomarker panels), which are selected by various statistical models and mathematical algorithms, for example, multiple logistic regression analysis. They fulfil the most appropriate diagnostic criteria for detection and staging of fibrosis and to a lesser extent for grading of fibrogenesis. In general, the panels consist of rather simple (standard) laboratory tests, which are only partially related to the mechanism of fibrogenesis, but subject to changes in the serum or plasma of fibrotic and cirrhotic patients (Table 2). Several of the parameters included in the more than 20 scores currently available have no pathophysiological relation to fibrogenesis. Some of them have an indirect relation, and only few parameters can be regarded as being directly related to fibrogenesis (Table 3). The parameters measured comprise those of necrosis, such as ALT and AST, coagulation-dependent tests, transport proteins, bilirubin and some ECM-parameters. Frequently, the reduction of platelet counts in cirrhotic patients is included. Cirrhosis-associated thrombopenia is based on sequestration of platelets in the enlarged spleen, reduced thrombopoietin synthesis in the metabolically insufficient liver and increased platelet consumption. Most of the scores were developed and tested in HCV-patients and, thus, their extrapolation to non-HCV-etiologies of fibrosis must be taken with caution. The reported data for sensitivity and specificity, respectively, are compiled in Table 2, but the values frequently have a great variance among the various studies. Most prevalent multiple parameter approaches of fibrosis are the fibrotest(tm) and of necro-inflammatory activity the actitest(tm), both commercially distributed by Biopredictive, Paris, France and LabCorp, Burlington, USA. They are based on γ-glutamyltransferase (γ-GT), total bilirubin, haptoglobin, α2-macroglobulin, apolipoprotein A1 and for actitest additionally on ALT [58–60]. The data of fibrotest and actitest are calculated with a patented artificial intelligence algorithm to give measures of fibrosis stage and necro-inflammatory grade (activity), respectively. The Wai-score based on AST, alkaline phosphatase and platelet count [61], the ELF-test based on TIMP-1, PIIINP, hyaluronan [62] and the Hepascore based on bilirubin, γ-GT, hyaluronan, α2-macroglobulin, age and gender [63]

Table 2 Class II Biomarkers of liver fibrogenesis

Index	Parameters	Chronic liver disease	Sensitivity (%)	Specificity (%)	References
PGAA-Index	Prothrombin time, γGT, apolipoprotein A1, α_2-macroglobulin	Alcohol	79	89	[112]
Bonacini-index	ALT/AST-ratio, INR, platelet count	HCV	46	98	[113]
Sheth-index	AST/ALT (De Ritis)	HCV	53	100	[114]
Park-index		HCV	47	96	[115]
PGA-Index	Prothrombin time, γGT, apolipoprotein A1	Mixed	91	81	[116] [117]
Fortunato-score	Fibronectin, prothrombin time, PCHE, ALT, Mn-SOD, β-NAG	HCV		94	[118]
Fibrotest (Fibro-score)	Haptoglobin, α_2-macroglobulin, apolipoprotein A1. γGT, bilirubin	HCV HBV	75	85	[58–60]
Pohl-score	AST/ALT-ratio, platelet count	HCV	41	99	[119]
Actitest	Fibrotest + ALT	HCV			[59]
Forns-index	Age, platelet count, γGT, cholesterol	HCV	94	51	[120]
Wai-index (APRI)	AST, platelet count	HCV	89	75	[61]
Rosenberg-score (ELF-score)	PIIINP, hyaluronan, TIMP-1	Mixed	90	41	[62]
Patel-index (FibroSpect)	hyaluronan, TIMP-1, α_2-macroglobulin	HCV	77	73	[121]
Sud-index (fibrosis probability-index, FPI)	age, AST, cholesterol, insulin resistance (HOMA), past alcohol intake	HCV	96	44	[122]
Leroy-Score	PIIINP, MMP-1	HCV	60	92	[123]
Fibrometer test	Platelet count, prothrombin index, AST, α_2-macro-globulin, hyaluronan, urea, age	Mixed	81	84	[124]
Hepascore	Bilirubin, γGT, hyaluronan, α_2-macroglobulin, age, gender	HCV	63	89	[63]
Testa-index	Platelet count / spleen diameter-ratio	HCV	78	79	[125]
FIB-4	Platelet count, AST, ALT, age	HCV/HIV	70	74	[126]
FibroIndex	Platelet count, AST, γ-globulin	HCV	38	97	[127]

Abbreviations: GGT, γ-glutamyltransferase; PIIINP, N-terminal pro-peptide of type III pro-collagen; TIMP, tissue inhibitors of metalloproteinases; MMP, matrix metalloproteinases; β-NAG, N-acetyl-β-glucosaminidase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; INR, international normalized ratio.

Table 3 Laboratory parameters included in multi-parameter scores (panels) and their potential pathogenetic link to fibrogenesis/fibrosis

Parameter	Potential pathobiochemical basis
Platelets (thrombocytes)	• Impaired synthesis due to reduced thrombopoietin production in diseased liver • Enhanced consumption in chronically inflamed liver by disseminated intravascular coagulation or immune mechanisms • Increased destruction in enlarged spleen, shortening of platelet life time
Prothrombin time (partially activated thromboplastin time)	• Measures activity/concentration of hepatogenic coagulation factors 1, 2, 5, 8–12, indicators of liver cell protein synthesis • Prolongation due to decreased production in liver cell insufficiency
Aspartate aminotransferase (AST)	• Parameter of liver cell necrosis (and apoptosis ?) • Leakage from cytosol and mitochondria into blood stream
Alanine aminotransferase (ALT)	• Parameter of liver cell necrosis (and apoptosis ?) • Leakage from cytosol into sinusoidal blood stream
γ-glutamyltransferase (γGT)	• Sensitive parameter of hepatobiliary diseases (cholestasis) • Induction by abuse of alcohol (ethanol) and certain drugs
Pseudo-cholinesterase (PCHE)	• Liver (hepatocyte)-specific enzyme • Parameter of anabolic liver cell insufficiency
Bilirubin	• Degradation product of haemoglobin removed by hepatocytes • Parameter of hepato-biliary diseases
α_2-macroglobulin	• High molecular mass glycoprotein synthesized in hepatocytes, which serves as • proteinase inhibitor and scavenger protein, acute-phase-protein • Binds TGF-β, CTGF(?) and other cytokines, involved in their clearance from circulation by hepatocytes
Hyaluronan (hyaluronic acid)	• Unsulfated, protein-free, highly polymerized glycosoaminoglycan, component of fibrotic matrix, synthesized by activated hepatic stellate cells • Important endogeneous ligand for Toll-like receptor TLR-4 of Kupffer cells and hepatic stellate cells
Cholesterol	• Impaired synthesis in hepatocytes by HMG-CoA-reductase in advanced liver insufficiency, no obvious link to fibrogenesis
Apolipoprotein A-I	• Component of HDL, up-regulation in and secretion by activated hepatic stellate cells, expression in hepatocytes, no obvious link to fibrogenesis
Aminoterminal pro-peptide of type III pro-collagen (PIIINP)	• Increased production of the N-terminal split product of type III pro-collagen during fibrogenesis
Tissue inhibitor of metallo-proteinases (TIMP-1)	• Up-regulation in fibrotic liver and in activated hepatic sellate cells, promotes progression of fibrosis through inhibition of matrix degradation
N-acetyl-β, D-glucosaminidase (β-NAG)	• Increased activity in liver and serum in acute and chronic-active liver injury, correlation with the grade of fibrogenic activity
Haptoglobin	• In hepatocytes synthesized acute-phase-protein, indicates inflammation but unspecific, scavenger protein for hemoglobin, antioxidans, no obvious link to fibrogenesis
HOMA, insulin resistance index	• Hyperinsulinemia (insulin resistance) is associated with rapid fibrosis progression in HCV, insulin stimulates hepatic stellate cells to collagen synthesis, glucose up-regulates CTGF/CCN2 and TGF-β
Fibronectin	• Matrix-associated plasma protein, increased expression in fibrotic conditions, up-regulation in activated hepatic stellate cells
Matrix metallo-proteinase-1 (MMP-1)	• Proteolytic enzyme involved in degradation, turnover and re-modelling of extracellular matrix

are further scores with up to now limited clinical application. In particular, the fibrotest was extensively evaluated and suggested as alternative to liver biopsy for estimation of the severity of chronic HCV infection. Fibrotest was recommended to be a better predictor than biopsy staging for HCV complications and death [60]. Recently, Fibrotest and Actitest were included into biomarkers for the prediction of liver steatosis (Steato-test™), alcoholic steato-hepatitis (ASH-test™), and non-alcoholic steato-hepatitis (NASH-test™) by supplementation with serum cholesterol, triglycerides, glucose (and AST for NASH-test) adjusted for age, gender and body mass index (BMI) [64, 65] (available from LabCorp, Burlington, USA). The diagnostic criteria elaborated in a large cohort of patients suggest Steato-test as a simple and non-invasive quantitative measure of liver steatosis and the NASH-test as a useful screening procedure for advanced fibrosis and NASH in patients with various metabolic syndromes [65]. It is proposed that these scores can reduce the need for liver biopsies. FibroMax™ (Biopredictive) was recently developed as a method of combined calculation of these fibrosis-related tests in a single procedure. Comparative evaluation of class II serum biomarker panels, however, could not highlight their clinical superiority [66]. Since only about 40% of the results were assigned to be correct, a fraction of about 50–70% was inaccurate with regard to the staging of fibrosis severity and a small fraction of results was even incorrect [66]. Thus, presently suggested multi-parameter approaches with class II fibrosis biomarker panels have to be taken with caution in clinical practice. A successful approach to improve the diagnostic accuracy of the panel markers in chronic HCV might be their stepwise combination [67]. By combining the sequential algorithms of AST to platelets ratio, Forns' index and fibrotest (Table 2) the diagnostic performance could be significantly improved resulting in a reduction of the need for liver biopsy by 50–70% [67]. However, biopsy as a 'base-line' diagnostic procedure cannot be completely avoided [68]. However, it should be emphasized that the combination of individually assessed parameters necessarily creates relative high variance due to the imprecision of each separate measurement [69]. Coefficients of variation range from series to series between 3% and 6% for common clinical-chemical parameters and from 4% to more than 12% for hyaluronan, PIIINP, and other matrix parameters. Furthermore and even more important is the lack of standardized assays for many of these parameters, which excludes the general use of cut-offs

and algorithms [69]. As an example, among the proteins included in fibrosis scores, only α2-macroglobulin and haptoglobin can be calibrated with the ERM-DA470 reference material (ERM = European reference materials, formerly CRM-470), which is accepted in Europe, US and Japan. Similarly, only a few clinical-chemical parameters are measured on the basis of IFCC (International Federation of Clinical Chemistry) standardization, several other parameters such as, γ-GT, hyaluronan, apolipoprotein A1, TIMP-1, MMP-1 are far away from an internationally standardized reference. In addition to pre-analytical variables, ethnic differences have to be considered. These limitations argue against a general, that is, worldwide acceptance of reported cut-offs and algorithms [69].

Genetic pre-disposition biomarkers

The pre-disposition to develop hepatic fibrogenesis is genetically complex and is the overall result of an interplay between genes and environment that is not simply following the characteristics of Mendelian disorders [70]. In addition, there are ethnic-dependent factors influencing the rate of progression and outcome of hepatic fibrogenesis. As a consequence, the 'fibrogenic traits' are genetically widely diverse in penetrance and progression. However, in the past there were several gene variations and polymorphisms identified that directly or indirectly increase the relative risk to develop hepatic fibrosis. In the Asian ancestry, the dependence on alcohol, which is one of the major 'Lifestyle injurious of the liver', was shown to be directly influenced by variations in the genes encoding alcohol dehydrogenase [71] and aldehyde dehydrogenase [72]. A similar association was found between the occurrence of a defined amino acid substitution (valine to alanine) within the mitochondrial-targeting sequence of manganese superoxide and the observed liver damage during long term alcohol abuse [73]. Although this modification does not modify alcohol consumption, the alanine-encoding allele is a major risk factor for severe alcoholic liver disease [73]. More recently, a similar functional polymorphism was found in the promoter region of the CD14 gene that causes higher serum levels of acute-phase proteins and which is a risk factor for advanced alcoholic liver disease in heavy drinkers [74].

In patients with chronic HCV infection, a functional genome-wide scan consisting of approximately 25,000 gene-centric single nucleotide polymorphisms

Table 4 Summary of genes associated with pre-disposition for liver fibrosis

Fibrogenic Mediators	Pre-disposition genes	References
Alcohol	Alcohol dehydrogenase	[71]
	Aldehyde dehydrogenase	[72]
	Manganese superoxide	[73]
	CD14	[74]
	Cytochrome P450IIE1	[128]
Hepatitis C virus	DDX5	[75]
	CPT1A	[75]
	Microsomal epoxide hydrolase	[129]
Growth factors/ Cytokines and their receptors	TGF-β1	[76, 79]
	IL-1 receptor	[130]
	IFN-γ	[81]
	TNFα	[131]
	IL-10	[132]
	Angiotensinogen	[76]
Chemokines and receptors	RANTES	[82]
	MCP-2	[82]
	CCR5	[82]
Serum lipoproteins	Apo E	[86, 133]
Immune- and complement system	CTLA-4	[83]
	TAP2	[134]
	Human leukocyte antigens (HLA)	[130]
	Complement factor C5	[84]
Iron metabolism	Haemochromatosis gene (*HFE*)	[87]

Abbreviations used are: ApoE, apolipoprotein E; CCR5, C-C motif receptor 5; CPT1A, carnitine palmitoyltransferase 1A; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; DDX5, DEAD box polypeptide 5; IFN-γ, interferon-γ; IL, interleukin; MCP-2, Macrophage chemoattractant protein 2; RANTES, Regulated upon Activation, Normal T cell Expressed and Secreted; TAP2, transporter associated with antigen processing 2; TNF-α, tumour necrosis factor-α.

(SNPs), two novel markers, located in the genes encoding DEAD box polypeptide 5 (DDX5) and carnitine palmitoyltransferase 1A (CPT1A) were significantly associated with advanced hepatic fibrosis [75].

Although, the precise impact of gene polymorphisms located within the coding region of the *TGFB1* gene is still controversially discussed [76–80] it becomes evident that the overall allelic frequencies and influence of individual gene variations on hepatic fibrogenesis is strongly dependent on other genetic factors that are fixed by ethnicity [79]. It is evident that any variation of this pro-fibrogenic cytokine that causes alterations in the biological activity (secretion, half-life) should have significant influence on the severity and progression of fibrogenesis.

Another cytokine sequence variant that was associated with the clinical outcome of hepatic fibrogenesis during chronic HCV infection is a T-to-A polymorphism at position +874 in the interferon (IFN)-γ gene [81]. In addition to cytokines, several modifications in chemokines with potent leukocyte activation and/or chemotactic activity and their receptors were identified to enhance the fibrogenic response of the liver. In Europeans, defined variations of the chemokines RANTES and MCP-2 and the chemokine receptor CCR5 were shown to influence the severity of fibrogenesis during HCV infection [82]. Moreover, an amino acid exchange within the cytotoxic T lymphocyte-associated antigen-4 gene (*CTLA-4*) encoding a molecule that is a vital negative regulator of T cell activation was linked to the susceptibility for primary biliary cirrhosis [83]. It becomes evident that also variants of the complement system, which is relentless activated during chronic HCV infection, cause differences in the genetic susceptibility for liver fibrosis. This was demonstrated in the identification of a quantitative trait locus on chromosome 2 carrying the complement factor C5 that was correlated with liver fibrogenesis in mice and humans [84]. However, it seems that this pro-fibrogenic and pro-inflammatory effect of C5 haplotypes is co-defined by levels of vitamin-D-binding protein in blood [85].

Moreover, several isoforms of serum lipoproteins that serve as systemic carriers for hepatitis viruses are known to induce the risk to develop more severe fibrosis. For instance, a specific association of hepatic fibrogenesis in patients suffering from HCV infection were found for a apolipoprotein E isoform [86]. Interestingly, it seems that this isoform specifically increases the risk for viral induced liver damage but not for other damages that were induced by non-viral causes.

Several independent studies have further shown that elevated levels of iron that cause iron deposition and chronic inflammation are independent risk factors for liver fibrosis and cirrhosis. The C282Y mutation of the haemochromatosis gene (*HFE*), for example, is associated with more advanced liver disease in chronic

HCV suggesting a role of *HFE* mutations (or higher overall concentration of iron) as primary risk factors for fibrogenesis and disease progression [87–89].

There are many other genes that are discussed in the literature as potential candidate genes influencing the pathogenesis or progression of chronic liver disease (Table 4). Some of them influence the metabolism and biological activity of substances with liver pathogenic attributes (*e.g.* alcohol, activity of viruses) while others act as additional risk factors (*e.g.* gene polymorphisms of cytokines and growth factors) corrupting the functionality of the liver. The progression rate of fibrosis and subsequent to cirrhosis varies widely among patients. It is tempting to speculate that this variation is not simply engendered by one of the gene polymorphisms mentioned above. It is more likely, that the susceptibility for fibrogenesis is generated by so called 'SNP signatures'. A recent report investigating the impact of 361 selected SNPs for assessment of the risk for cirrhosis have shown that a cirrhotic risk signature (CRS) containing seven predictive SNPs can identify Caucasian patients with chronic hepatic C infection at high risk for cirrhosis [90]. In this study, the area-under-the receiver-operating-characteristic (ROC) curves of the CRS was 0.75 in a Caucasian training cohort and 0.73 in a validation cohort suggesting that CRS is a better predictor than clinical factors in differentiating high-risk versus low-risk for cirrhosis in Caucasian CHC patients [90].

Although it is tempting to speculate that some of the gene markers that were reported so far as genetic pre-disposition markers for hepatic fibrogenesis have great potential, none of them has actual relevance in routine diagnosis or prognosis of fibrosis susceptibility. Further, large-scale, well-designed association studies will prove the efficacy of potential new genetic tests.

Future developments

Growing understanding of the pathogenesis of hepatic fibrosis indicates potentially powerful non-invasive (blood) biomarkers of hepatic fibrogenesis and fibrosis (Table 5). CTGF/CCN2 was already mentioned as an important, pluripotent downstream modulator of growth factors, in particular of TGF-β, and was found to be up-regulated by TGF-β in hepatocytes. Although most of CTGF will only have a defined paracrine function in fibrogenic tissue, a certain fraction spillsover into the circulation, resulting in elevated serum concentrations during active fibrogenesis [55]. Preliminary data justify the optimism that the circulating level of CTGF might be an objective and sensitive readout of ongoing fibrogenesis in necro-inflammatory liver tissue.

The description of bone-marrow-derived fibrocytes might offer new approaches not only for the understanding of the pathogenesis, but also for the diagnosis of liver fibrosis. Fibrocytes are circulating progenitor cells (CD34 positive) of haematopoietic origin (CD45 positive) capable of differentiating into diverse mesenchymal cell types [21]. The additional markers of fibrocytes, *i. e.*, positivity of type I collagen and the CXCR4 chemokine expression can be used to quantitate this special sub-population of circulating leucocytes in the buffy coat or even in the circulation applying quantitative PCR and/or flow cytometry. The determination of the colony-stimulating factors M-CSF, G-CSF and granulocyte macrophage-colony stimulating factor (GM-CSF), which are increasingly expressed in fibrotic liver tissue and elevated in serum [91, 92], are possibly involved in the mobilisation of fibrocytes from the bone marrow and their homing in the liver during fibrogenesis. They might be further candidates for diagnostic evaluation.

A new, but presently still controversial aspect of fibrogenesis is EMT as outlined above. EMT is governed by the balance of TGF-β (pro-EMT) and its antagonist, that is, BMP-7 (anti-EMT). In addition to its anti-EMT effect, BMP-7 was shown to have anti-apoptotic and anti-inflammatory activities. Thus, the measurement of BMP-7 alone or even in relation to TGF-β in serum might reflect the activity of fibrogenesis and, hence, the velocity of fibrotic organ transition. Elevated BMP-7 levels and up-regulated expression in hepatocytes of cirrhotic livers *in situ* were reported [93].

Xylosyltransferase, a key enzyme in the biosynthesis of glycosaminoglycans in proteoglycans, was shown to have increased activities in serum of patients with connective tissue diseases, for example, systemic sclerosis, osteoarthritis and pseudoxanthoma elasticum. With highly sensitive and automated methods (high-performance liquid chromatography [HPLC]-tandem mass spectrometry) measurements in large cohorts of liver fibrotic patients seem to be possible [94]. Since HSC in fibrotic liver tissue (MFB) have a greatly stimulated proteoglycan synthesis [95, 96] xylosyltransferase activity in serum might also be a promising class I biomarker of fibrogenesis.

Table 5 Future candidate biomarkers of non-invasive diagnosis and follow-up of liver fibrogenesis

Biomarker	Specimen	Assay technology	Pathobiochemical basis
CTGF	Serum	Immunoassay	TGF-β induced expression in and secretion by hepatocytes and hepatic stellate cells
Fibrocytes	Blood, buffy coat	Flow cytometry of CD34$^+$, CD45$^+$, Coll I$^+$ cells QPCR	Supplementation of local fibroblasts at site of liver injury by bone-marrow derived fibrocytes
BMP-7	Serum	Immunoassay	Antagonist of TGF-β, inhibitor of EMT
G-CSF GM-CSF M-CSF	Blood	Immunoassays	Mobilization of bone-marrow derived fibrocytes
^{13}C-Methace-tin breath test	Expiratory air	Miniaturized spectroscopy and continuous breath sampling	Reflects hepatic microsomal function of CYP450 1A2
Proteomics	Serum	Mass spectrometry (MS)	Fibrosis-specific serum protein profiles
Glycomics	Serum	Adaptation of DNA-sequencer/ fragment analyser technology to profiling of desialylated N-linked oligo-saccharides	Fibrosis-specific profiles of desialylated serum protein linked oligosaccharides (N-glycans)
Xylosyl-transferase (EC 2.4.2.26)	Serum	LC-MS/MS	Key enzyme in the biosynthesis of glycosaminoglycan chains in proteoglycans, *e. g.* in hepatic stellate cells

Recently proposed point of care non-invasive ^{13}C-methacetin breath testing for identifying a significant inflammation and fibrosis or NAFLD provides optimistic data for significant fibrosis (METAVIR > 2) with sensitivities and specificities of 96% and 86%, respectively [97]. This liver function test measures microsomal activity of cytochrome P4501A2, which is related to inflammation and fibrosis. Evaluation in large cohorts is necessary.

Further successful developments could emerge from serum proteom profiling [98] and from total serum protein glycomics, that is, the pattern of N-glycans [99]. It was reported that a unique serum proteomic finger print is identified in the sera of patients with fibrosis, which enables differentiation between different stages of fibrosis and a prediction of fibrosis and cirrhosis in patients with a chronic hepatitis B infection [98]. Specificities and sensitivities and accuracy of prediction of cirrhosis are around 89%. Similarly, N-glycan profiling can distinguish between compensated cirrhosis from non-cirrhotic chronic liver diseases with sensitivity and specificity of 79% and 86%, respectively [99]. Besides pushing forward new parameters, the refinement of already existing class I biomarkers will be promising. As an example, the differentiation of low and high molecular weight fractions of hyaluronan, specific immunoassays of the core-proteins of proteoglycans synthesized in activated stellate cells (*i.e.* biglycan, decorin), and TGF-β-related components (*i.e.* latency associated protein of TGF-β and latent TGF-β-binding protein) are rational candidates of new or refined biomarkers. The evaluation of all these non-invasive diagnostic tools remains a complicated matter because of the limitations of the presently available 'gold standard' liver biopsy described above [6]. Although the histologic evaluation of biopsy specimens provides a unique source of grading and staging of inflammation, steatosis, fibrosis, cirrhosis and neoplasia considerable sampling variability, inter and intraobserver variance and insensitive semiquantative numerical scores are major analytical drawbacks. Recently developed methods of quantitative morphometric image analysis partially overcome these limitations [100], but the diagnostic power remains dependent on good sample quality [100] and expertise of the observer. Thus, a tissue cyinder of at least 25 mm length is necessary to evaluate fibrosis correctly [4].

Supplementation of all these techniques by modern high resolution or even molecular imaging analyses would be extremely helpful in the consolidation of objective and valid non-invasive biomarkers of diagnosis

and follow-up of fibrogenic (liver) disease. These include ultrasound [101, 102], magnetic resonance imaging [103] and transient elastography [104]. The latter method (FibroScan^{TM}), Echosens, France), a specially adapted pulse-echo ultrasound technique, uses the principle of one-dimensional transient elastography to measure liver stiffness. This method appears as a reliable tool to detect significant fibrosis or cirrhosis in patients with chronic HCV [105, 106] and was even superior to fibrotest [107]. FibroScan may be particular useful to monitor patients longitudinally after a baseline biopsy has been performed [108]. The combination with serum fibrosis markers further improves the accuracy to the diagnosis of significant fibrosis (Metavir F≤2) [109, 110]. In conclusion, currently available type I and II serum biomarkers should be used with caution, because neither single nor panel markers fulfil the requirements of an ideal non-invasive biomarker of fibrosis [111], that is, analytical simplicity allowing performance in any laboratory, standardization of the test system and calibrators allowing comparison between the laboratories over a long period, cost effectiveness, specificity for the liver and the disease, clear association with the stage of fibrosis or grade of fibrogenesis and independency of the etiology of the fibrosis (*e.g.* alcoholic, HCV, B and others). Even the relative best and most extensively evaluated type I (*i.e.* hyaluronan) and type II (*i.e.* fibrotest, actitest) serum biomarkers do not meet the criteria of an ideal marker. Further detailed insight into the mechanism of liver fibrosis and improvement of analytical techniques will result in new approaches for non-invasive assessment of fibrosis with biochemical or physical means. In addition, the analysis of genetic pre-disposition markers or the determination of special SNPs signatures that are associated with the severity of fibrosis will potentially complement serum analytic, proteom profiling, and the non-invasive fibrosis staging testing.

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