Non-invasive biomarkers for monitoring the fibrogenic process in liver: A short survey

Axel M Gressner, Chun-Fang Gao, Olav A Gressner

Abstract

The clinical course of chronic liver diseases is significantly dependent on the progression rate and the extent of fibrosis, i.e. the non-structured replacement of necrotic parenchyma by extracellular matrix. Fibrogenesis, i.e. the development of fibrosis can be regarded as an unlimited wound healing process, which is based on matrix (connective tissue) synthesis in activated hepatic stellate cells, fibroblasts (fibrocytes), hepatocytes and biliary epithelial cells, which are converted to matrix-producing (myo-)fibroblasts by a process defined as epithelial-mesenchymal transition. Blood (non-invasive) biomarkers of fibrogenesis and fibrosis can be divided into class I and class II analytes. Class I biomarkers are those single tests, which are based on the pathophysiology of fibrosis, whereas class II biomarkers are mostly multiparametric algorithms, which have been statistically evaluated with regard to the detection and activity of ongoing fibrosis. Currently available markers fulfill the criteria of ideal clinical-chemical tests only partially, but increased understanding of the complex pathogenesis of fibrosis offers additional ways for pathophysiologically well based serum (plasma) biomarkers. They include TGF-β-driven marker proteins, bone marrow-derived cells (fibrocytes), and cytokines, which govern pro- and anti-fibrotic activities. Proteomic and glycomic approaches of serum are under investigation to set up specific protein or carbohydrate profiles in patients with liver fibrosis. These and other novel parameters will supplement or eventually replace liver biopsy/histology, high resolution imaging analysis, and elastography for the detection and monitoring of patients at risk of developing liver fibrosis.

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Key words: Biochemical markers; Diagnostic validity; Liver fibrosis; Monitoring; Multiparametric algorithms; Non-invasive diagnostic tools

INTRODUCTION

Tissue fibrosis is characterized by the excess deposition of extracellular matrix (ECM) involving molecular and histological re-arrangement of various types of collagens, proteoglycans, structural glycoproteins and hyaluronan (Figure 1). It is a hallmark of liver cirrhosis and contributes significantly to the deleterious outcome of chronic liver diseases[1]. The deposition of ECM in the space of Disse (perisinusoidal fibrosis) between the sinusoidal surface of hepatocytes and the endothelial cell layer of liver sinusoids, the generation of (incomplete) subendothelial basement membranes, and the strangulation of hepatocytes by surrounding matrix impair not only the blood flow through the organ, but also the biosynthetic function of hepatocytes and the clearance capability of these and other cell types[2].

The molecular pathogenesis of the fibrotic transition of liver parenchyma turns out to be a multi-faceted process largely due to the activation of resting, vitamin A-storing stellate cells to matrix-producing myofibroblasts[3,4] in the immediate neighbourhood of hepatocytes, to the phenotypic switch of hepatocytes and bile duct epithelial
cells to fibroblasts termed epithelial-mesenchymal transition (EMT)\cite{5-7}, and to the influx of bone marrow-derived cells (fibrocytes) reaching the liver via the systemic circulation\cite{8,9} (Figure 2). The fractional contribution of these pathways to fibrosis depends on the underlying disease and probably on the stage of the fibrotic transition\cite{2}. The activation of stellate cells results from interaction with damaged hepatocytes, activated Kupffer cells, disintegrated platelets and various subfractions of leucocytes. Among the cytokines involved in the pathogenetic processes, TGF-β plays a dominant role, but PDGF, endothelin-1, VEGF, and others also contribute significantly. Antagonistic (antifibrotic) mediators might also exist among which bone morphogenetic protein (BMP)-7 plays an important role, e.g. in the inhibition of EMT-derived fibroblasts\cite{10}. Therefore, the development of non-invasive, objective and quantitative serum- or plasma-based biomarkers of fibrogenesis is an important goal, which can be approached by the assessment of two, principally different lines of blood-borne (non-invasive) analytes: Class I and class II serum fibrosis markers.

**CLASSIFICATION OF CIRCULATING BIOMARKERS OF FIBROSIS**

Class I fibrosis biomarkers are pathophysiologically derived from ECM turnover and/or from changes of the fibrogenic cell types, in particular hepatic stellate cells (HSC) and (myo-)fibroblasts\cite{9}. They should reflect the activity of the fibrogenic and/or fibrolytic process and, thus, remodelling of ECM. These biomarkers do not indicate the extent of connective tissue deposition, i.e. the stage of fibrotic transition of the organ. Frequently, they involve costly laboratory tests and are the result of translation of fibrogenic mechanisms into clinical application. Thus, their selection is hypothesis-driven.

Class II fibrosis 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

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**Figure 1** Components of the extracellular matrix (connective tissue) of the fibrotic liver and their major changes. The binding of glycosaminoglycans (GAG) to the respective core proteins (CP) of proteoglycans (PG) are shown. BM: Basement membranes; FACIT: Fibril-associated collagens with interrupted triple-helices.
reflect ECM metabolism or fibrogenic cell changes. Their pathobiochemical relationship with fibrogenesis is indirect if at all. Thus, their selection is not hypothesis-driven, but empiric. The markers are standard laboratory tests and are if at all. Thus, their selection is not hypothesis-driven, but empiric. The markers are standard laboratory tests and are integrated into multiparametric panels.

In general, both types of serum biomarkers follow different pathophysiological concepts. 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 fibrosis biomarkers**

These biomarkers are components of the connective tissue (matrix) increasingly expressed by activated hepatic stellate cells (HSC) and (myo-)fibroblasts, have a delayed clearance by Kupffer cells or sinusoidal endothelial cells in the liver due to metabolic dysfunction and/or hemodynamic bypasses, or are increasingly expressed mediators of fibrogenesis such as TGF-β. Taken together, of the several procollagen and collagen fragments proposed, only the N-terminal propeptide of type III procollagen (PⅢNP) has reached a limited clinical application, but not widespread acceptance. Sensitivities of about 76%-78% and specificities of 71%-81% have been reported, which can be increased up to 88%, if combined with additional collagen fragment markers. It should be emphasized that PⅢNP is not a liver-specific biomarker. Similarly, structural glycoproteins (e.g. undulin, tenasin), biosynthetic (e.g. prolyl hydroxylase) or catabolic enzymes (e.g. matrix metalloproteinases) of collagen and other ECM-components have not been convincing in the detection, grading, and staging of fibrosis (Table 1). Several studies have shown that hyaluronic acid (hyaluronan) is currently the best class I biomarker of fibrosis having an area under the receiver operating characteristics (AUC) of 0.97, a sensitivity of 86%-100%, and a specificity of about 88% in a recent investigation of cirrhosis due to non-alcoholic fatty liver disease and other aetiologies. Since the negative predictive value of hyaluronan at a cut off value of 60 µg/L is much higher (98%-100%) than the positive predictive value (61%), the main utility of serum hyaluronan lies in its ability to exclude advanced fibrosis and cirrhosis. Its
stimulated synthesis in activated HSC, secretion into the sinusoidal blood stream, and short half life of 2-9 min in the circulation are good suppositions for a valid fibrosis biomarker. Laminin was reported to be a predictor of portal hypertension since significantly elevated concentrations were found under these conditions \[15\]. TGF-β concentration in plasma is elevated in and correlates with the severity of liver disease and is suggested to be a non-invasive biomarker of fibrosis. However, the significant correlation with AST and ALT activity \[16\] and the pathobi-chemical finding that substantial amounts of TGF-β are localized in hepatocytes and released into the medium if hepatocytes are permeabilized \[17\] suggest that the elevation of TGF-β is a marker of necrosis instead of fibrogenesis.

Preliminary studies point to connective tissue growth factor (CTGF/CCN2) in serum as an innovative class I biomarker of fibrogenesis \[18\]. This 38 kDa protein is synthesized not only in HSC, but also in hepatocytes where the expression and secretion is strongly dependent on TGF-β \[15,19\]. Accordingly, the expression of the TGF-β down-stream mediator CTGF in fibrotic liver tissue is up-regulated and its concentration in blood is elevated if fibrogenesis is occurring. There is a correlation between CTGF levels and fibrogenesis, because the levels decrease in fully developed, end-stage cirrhosis, compared to fibrosis. The AUCs for fibrosis vs control and cirrhosis vs control were calculated to be 0.955 and 0.887, respectively, the sensitivities 100% and 84%, respectively, the specificities 89% and 85%, respectively \[18\]. These criteria suggest that CTGF is a potentially valuable class I biomarker of active fibrogenesis.

Recently, the glycoprotein YKL-40 (“chondrex”, molecular mass 40 kDa), which is likely a growth factor for fibroblasts and endothelial cells, was shown to be

Table 1  Class I biomarkers of liver fibrogenesis

| Specimen                  | Method          |
|---------------------------|-----------------|
| Serum         | Urine | Liver biopsy |
| Extracellular matrix-related enzymes |     |             |
| Enzyme      |     |             |
| Prolyl hydroxylase       | +   | -   | +   | Radioenzymatic, RIA |
| Monoamine-oxidase        | +   | -   | (+) | Enzymatic          |
| Lysyl oxidase            | +   | -   | +   | RIA                 |
| Lysyl hydroxylase        | +   | -   | -   | RIA                 |
| Galactosylhydroxylysylglucosyltransferase | +   | -   | +   | RIA                 |
| Collagen peptidase       | +   | -   | +   | Enzymatic          |
| N-Acetyl-β-D-glucosaminidase | +   | +   | +   | Enzymatic          |
| Collagen fragments and split products |     |             |
| Type of collagen         |     |             |
| Type I -procollagen      |     |             |
| N-terminal propeptide (PINP) | +   | -   | +   | ELISA             |
| C-terminal propeptide (PICP) | +   | -   | +   | RIA                 |
| Type III -procollagen    |     |             |
| Intact Procollagen       |     |             |
| N-terminal propeptide (PⅠNP) | +   | -   | -   | RIA                 |
| Complete propeptide (Col 1-3) | +   | -   | -   | RIA                 |
| Globular domain of Propeptide (Col-1) | +   | -   | -   | RIA                 |
| Type IV -Collagen        |     |             |
| NCI-fragment (C-terminal) |     |             |
| crosslinking domain (PIV) | +   | +   | -   | ELISA, RIA         |
| 7S domain (7S Collagen)  | +   | +   | -   | RIA                 |
| Type VI-Collagen         | +   | +   | +   | RIA                 |
| Glycoproteins and matrix-metalloproteinase (inhibitors) |     |             |
| Marker                   |     |             |
| Laminin, PI-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) | +   | -   | -   | ELISA             |
| sVCAM-1 (soluble vascular cell adhesion molecule, sCD106) | +   | -   | -   | ELISA             |
| Glycosaminoglycans       |     |             |
| Marker                   |     |             |
| Hyaluronic acid (Hyaluronan) | +   | -   | -   | Radioligand assay ELISA |
| Molecular mediators      |     |             |
| Marker                   |     |             |
| Transforming growth factor β (TGF-β) | +   | -   | +   | ELISA             |
| Connective tissue growth factor (CTGF/CCN2) | +   | ?   | +   | ELISA             |

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strongly expressed in human liver tissue\textsuperscript{[21]}. In particular, HSC contain YKL-40 mRNA. Several studies have found elevated YKL-40 concentrations in sera of patients with liver diseases. A sensitivity and specificity of around 80\% and an AUC of 0.81 for fibrosis have been reported for HCV-patients\textsuperscript{[23]}, for those with alcoholic liver disease, a specificity of 88\% and a low sensitivity of 51\% were calculated\textsuperscript{[23]}. Serum concentrations of this protein correlated with other ECM products secreted by HSC and fibroblasts, e.g. P\textsuperscript{N\textsuperscript{II}}NP, hyaluronan, MMP-2, and TIMP-1. It is claimed that YKL-40 concentrations reflect the degree of liver fibrosis but extensive clinical evaluation is still required and other inflammatory diseases as potential causes of YKL-40 elevations have to be excluded. In addition, the expression of this protein is not restricted to the liver, but occurs in chondrocytes (synovial fluid), bone cells, vascular smooth muscle cells and therefore non-specific to the liver\textsuperscript{[21]}.

### Class II fibrosis biomarkers

This category comprises a rapidly increasing, wide variety of biochemical scores and multiparameter combinations (biomarker panels), which are selected by various statistical models and mathematical algorithms, e.g. multiple logistic regression analysis. They fulfil the most appropriate diagnostic criteria for the 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 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 a few parameters can be regarded as being directly related to fibrogenesis. 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. Most prevalent are the Fibrotest\textsuperscript{TM} and for necro-inflammatory activity the Actitest\textsuperscript{TM} (Biopredictive, Paris, France)\textsuperscript{[25]}. These tests are based on \(\gamma\)-glutamyl-transfase (\(\gamma\)-GT), total bilirubin, haptoglobin, \(\alpha\)-macroglobulin, apolipoprotein A1, and for the Actitest additionally on alanine-aminotransferase; ALT: Alanine aminotransferase; AL: Alanine aminotransferase; INR: International normalized ratio.

| Class II biomarkers of liver fibrogenesis |
|-----------------------------------------|
| Index | Parameters | Chronic liver disease | Sensitivity (%) | Specificity (%) |
|---|---|---|---|---|
| PGAA-Index | Prothrombin time, \(\gamma\)-GT, apolipoprotein A1, \(\alpha\)-macroglobulin | Alcohol | 79 | 89 |
| Boncini-Index | ALT/AST-ratio, INR, platelet count | HCV | 46 | 98 |
| Sheth-Index | AST/ALT (De Ritis) | HCV | 53 | 100 |
| Park-Index | Prothrombin time, \(\gamma\)-GT, apolipoprotein A1 | Mixed | 91 | 81 |
| PGA-Index | Prothrombin time, \(\gamma\)-GT, apolipoprotein A1 | HCV | 47 | 96 |
| Fortunato-Score | Fibroten, prothrombin time, PCH, ALT, Mn-SOD, \(\beta\)-NAG | HCV | 94 | |
| Fibrotest (Fibro-Score) | Haptoglobin, \(\alpha\)-macroglobulin, apolipoprotein A1, \(\gamma\)-GT, bilirubin | HCV | 75 | 85 |
| Pohl-Score | ALT/AST-ratio, platelet count | HCV | 41 | 99 |
| Actitest | Fibrotest + ALT | HCV | | |
| Forns-Index | Age, platelet count, \(\gamma\)-GT, cholesterol | HCV | 94 | 51 |
| Wai-Index | AST, platelet count | HCV | 89 | 75 |
| (APRI) | | | | |
| Rosenberg-Score (ELF-Score) | P\textsuperscript{N\textsuperscript{II}}NP, hyaluronan, TIMP-1 | Mixed | 90 | 41 |
| Patel-Index (FibroSpect) | Hyaluronan, TIMP-1, \(\alpha\)-macroglobulin | HCV | 77 | 73 |
| Sud-Index (fibrosis probability-index, FPI) | Age, AST, cholesterol, insulin resistance (HOMA), past alcohol intake | HCV | 96 | 44 |
| Lary-Score | P\textsuperscript{N\textsuperscript{II}}NP, MMP-1 | HCV | 60 | 92 |
| Fibrometer test | Platelet count, prothrombin index, AST, \(\alpha\)-macro-globulin, hyaluronan, urea, age | Mixed | 81 | 84 |
| Hepscore | Bilirubin, \(\gamma\)-GT, hyaluronan, \(\alpha\)-macroglobulin, age, gender | HCV | 63 | 89 |
| Testa-Index | Platelet count/spleen diameter-ratio | HCV | 78 | 79 |
| FIB-4 | Platelet count, ALT, age | HCV/HIV | 70 | 74 |
| FibroIndex | Platelet count, AST, \(\gamma\)-globulin | HCV | 38 | 97 |

\(\gamma\)-GT: \(\gamma\)-glutamyltransferase; P\textsuperscript{N\textsuperscript{II}}NP: N-terminal propeptide of type \(\alpha\) procollagen; TIMP: Tissue inhibitors of metalloproteinases; MMP: Matrix metalloproteinases; \(\beta\)-NAG: N-acetyl-\(\beta\)-glucosaminidase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International normalized ratio.
Table 3 Future candidate biomarkers of non-invasive diagnosis and follow-up of liver fibrogenesis

| Biomarker   | Specimen     | Assay technology                          | Pathobiological 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       | Blood        | Immunoassays                              | Mobilization of bone-marrow derived fibrocytes                                      |
| GM-CSF      |              |                                           |                                                                                      |
| M-CSF       |              |                                           |                                                                                      |
| Proteomics  | Serum        | Mass spectrometry (MS)                    | Fibrosis-specific serum protein profiles                                           |
| Glycosomics | Serum        | Mass spectrometry (MS)                    | Fibrosis-specific profiles of desialylated serum protein linked oligosaccharides (N-glycans) |
| Xylosyl-transferase (EC 2.4.2.26) | Serum | Adaptation of DNA-sequencer/fragment analyzer technology to profiling of desialylated N-linked oligo-saccharides (N-glycans) | Key enzyme in the biosynthesis of glycosaminoglycan chains in proteoglycans, e.g. in hepatic stellate cells and hepatocytes |

A growing understanding of the pathogenesis of hepatic fibrosis has indicated potentially powerful non-invasive (blood) biomarkers of hepatic fibrogenesis and fibrosis (Table 3). CTGF/CCN2 was already mentioned as a pluripotent downstream modulator of TGF-β, and was found to be up-regulated by TGF-β in hepatocytes. Although most CTGF will only have a defined paracrine function in fibrogenic tissue, a certain fraction spills over into the circulation, resulting in elevated serum concentrations during active fibrogenesis[18]. The circulating level of CTGF might be an objective and sensitive measure of ongoing fibrogenesis in necro-inflammatory liver tissue.

Bone-marrow-derived fibrocytes might offer new approaches not only for understanding the pathogenesis, but also for the diagnosis of liver fibrosis. Fibrocytes are circulating progenitor cells (CD34 positive) of hematopoietic origin (CD45 positive) capable of differentiating into diverse mesenchymal cell types[33]. The additional markers of fibrocytes, i.e. positivity of type 1 collagen and the CXCR4 chemokine expression can be used to quantitate this special sub-population of circulating leucocytes applying quantitative PCR and/or flow cytometry. The determination of the colony stimulating factors M-CSF, G-CSF, and GM-CSF, which are increasingly expressed in fibrotic liver tissue and elevated in serum[34], are possibly involved in the mobilisation of fibrocytes from the bone marrow and their homing in the liver during fibrogenesis. These factors may be further candidates for diagnostic evaluation.

A new, but currently still controversial aspect of fibrogenesis is epithelial-mesenchymal transition (EMT) of hepatocytes and biliary epithelial cells, respectively, to (myo-)fibroblasts[3]. EMT is governed by the balance of TGF-β (pro-EMT) and its antagonist, i.e. 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[17].

Xylosyltransferase (XT), a key enzyme in the biosynthesis of glycosaminoglycans in proteoglycans, was shown to have increased activities in the serum of patients with connective tissue diseases. With HPLC-tandem mass spectrometry, measurements in large cohorts of liver fibrotic patients may be possible[18]. Since HSC in fibrotic liver tissue (myofibroblasts) have a greatly stimulated proteoglycan synthesis[19], XT activity in serum might be a promising class I biomarker of fibrogenesis.

Further successful developments could emerge from serum proteome profiling[34] and from total serum protein glycomics, i.e. the pattern of N-glycans[41]. It was reported that a unique serum proteome fingerprint is powerful enough (accuracy > 90%) to differentiate between various stages of fibrosis and to allow prediction of fibrosis and cirrhosis in patients with a chronic hepatitis B infection[40]. Specificities, 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 a sensitivity and specificity of 79% and 86%, respectively.[41]

Supplementation of all these laboratory tests by modern high resolution or 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) diseases. 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[39], i.e. analytical simplicity allowing performance in any laboratory, standardization of the test system and calibrators allowing comparison between 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 aetiology of fibrosis. Even the 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 improvements in analytical techniques will result in new approaches for the non-invasive assessment of fibrosis with biochemical or physical means.

In addition, genetic markers linked with the progression rate of fibrosis will become important diagnostic and prognostic tools for patients with liver fibrosis.

CONCLUSION

Non-invasive evaluation of the fibrogenic response of the chronically injured liver has made considerable progress over the past few years, in particular over the last three years multiple algorithms based on a combination of more or less routine parameters have been suggested frequently. A rigorous, independent and widespread evaluation of the utility of these panels in the diagnosis and follow-up of chronic liver diseases is still needed for a final decision and the recommendation for use in routine clinical practice. Novel single biochemical markers have been suggested, but there putative diagnostic value in clinical practice is far from defined. The fundamental problem in the evaluation of existing and novel non-invasive parameters lies in the limited validity of the present diagnostic “gold standard”, i.e. histology of liver biopsy specimens. Perhaps new developments in highly sensitive and tissue-specific scanning techniques of the liver will solve this problem. These procedures will then be suitable for the correct validation of effective antifibrotic treatments.

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