



REVIEW

# Non invasive fibrosis biomarkers reduce but not substitute the need for liver biopsy

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## Abstract

Chronic liver diseases are very common worldwide, particularly those linked to viral hepatitis and to alcoholic and non-alcoholic fatty liver. Their natural history is variable and long-term evolution differs in individual patients. Optimised clinical management of compensated chronic liver diseases requires precise definition of the stage of liver fibrosis, the main determinant of prognosis and of most therapeutic decisions. Liver biopsy is the gold standard for assessment of hepatic fibrosis. However, it is invasive with possible complications, costly and prone to sampling errors. Many non-invasive markers of liver fibrosis have been recently proposed and assessed in the clinical setting as surrogates of liver biopsy. Direct markers are based on biochemical parameters directly linked to fibrogenesis while indirect markers use simple or more sophisticated parameters that correlate with liver fibrosis stages. Non-invasive markers of liver fibrosis have been tested in different forms of chronic liver disease and showed variable diagnostic performance, but accuracy rarely was above 75%-80%. Better results were obtained when markers were combined. On this line, we have recently proposed a set of algorithms that combine sequentially indirect non-invasive markers of liver fibrosis, reaching 90%-95% diagnostic accuracy with significant reduction in the need for liver biopsy. Based on available evidence, it can be anticipated that non-invasive markers of liver fibrosis and their combined use will soon become a most useful tool in the clinical management of many forms of chronic liver disease. However, their implementation is expected to reduce, but not to completely eliminate, the need for liver biopsy.

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**Key words:** Chronic liver diseases; Hepatic fibrosis; Liver biopsy; Non invasive fibrosis markers

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## INTRODUCTION

Chronic infection with the hepatitis B (HBV) and C (HCV) viruses and alcoholic and non-alcoholic steatohepatitis are the main causes of chronic and progressive liver disease leading to cirrhosis, end-stage liver disease, and hepatocellular carcinoma worldwide, with a predominant role of hepatitis B in the Middle and Far East regions and of hepatitis C and steatohepatitis in the Western Countries. These different etiologic forms of chronic liver disease (CLD) have a common histopathological pathway that is the formation and accumulation of fibrosis leading to the development of progressive distortion of the hepatic architecture that is the hallmark of evolution to cirrhosis. Natural history studies indicate that advanced fibrosis and cirrhosis develop in about 20%-40% of patients with chronic hepatitis B or C and in a similar proportion of those with alcoholic or non alcoholic steatohepatitis<sup>[1-4]</sup>. Development of fibrosis is a step-by-step process starting from minimal fibrosis limited to the portal tracts, followed by more extensive fibrosis with septa expanding into the liver parenchyma, that can form bridges between two portal tracts or portal tracts and central veins, eventually ending in complete cirrhotic nodules. This type of progression may take years or decades to fully develop, and staging of hepatic fibrosis is therefore of paramount clinical importance for the prognostic assessment in the individual patient. In patients with chronic viral hepatitis precise definition of the hepatic fibrosis stage is the most important parameter to assess the risk of disease progression and to decide the need for immediate antiviral therapy. This is particularly true for those patients with chronic viral hepatitis or alcoholic or non-alcoholic fatty liver who are still in a well compensated phase and have no overt clinical or laboratory signs of cirrhosis. In these patients liver biopsy represents the gold standard for evaluating presence, type and stage of liver fibrosis. This procedure, however, is invasive, costly and difficult to standardise. In recent years there has been increasing interest in the possibility of identifying and describing liver fibrosis by using non invasive, surrogate markers measurable in the peripheral blood and many of such tests have been reported in the literature. This review is

aimed to describe the different non invasive markers and methods that have been proposed for the assessment of liver fibrosis, to discuss their advantages and limits and to suggest a rational use in clinical practice.

## HISTOLOGICAL CLASSIFICATION OF CHRONIC LIVER DISEASE

The old classification of chronic hepatitis made a rough grading distinction between milder and more severe forms of liver disease. More recently, the new insights in the etiology and therapy of CLDs, particularly viral hepatitis, has led to a revised classification, aimed to describe and quantify in more details necroinflammation and fibrosis. Several semiquantitative scoring systems have been proposed to measure the activity grade of inflammation and to stage the amount and type of fibrosis in the liver. Scoring systems specifically designed for chronic viral hepatitis are the histological activity index proposed in 1981 by Knodell *et al.*<sup>[5]</sup>, the Ishak's score<sup>[6]</sup> and the METAVIR scoring system<sup>[7]</sup>.

The histological activity index is based on the evaluation of four parameters: periportal necrosis (score of 1-10), parenchymal damage (score of 0-4), portal inflammation (score of 0-4) and fibrosis (score of 0-6). The cumulative score therefore ranges from 0 to 18 to describe the overall histological activity. The limitation of this scoring system is that necro-inflammation (grading) scores are cumulated with the fibrosis (staging) scores, while these parameters describe different lesions and clearly have different prognostic implications. The Ishak's system is a revised version of the histological activity index, and describes the activity grade and the fibrosis stage as two separate items. Liver fibrosis is here classified in absent (0), mild (1-2), moderate (3-4) and severe/cirrhosis (5-6). The METAVIR scoring system for liver fibrosis, frequently used in recent times particularly for chronic hepatitis C, is described in details in Table 1. All these scoring systems have some limits, being semiquantitative, not linear and prone to intra- and inter-observer variation and to sampling variability<sup>[8]</sup>. They have been more validated for clinical use with some but not all the different etiological forms of CLD that are characterised by progressive fibrosis leading to cirrhosis.

## LIVER BIOPSY: PROS VS CONS

For many years liver biopsy has been considered the golden standard for the evaluation of liver fibrosis staging. Liver biopsy has the advantage of allowing to obtain information not only on fibrosis, but also on many useful parameters, such as inflammation, necrosis, steatosis, hepatic iron and so on. Furthermore, it allows to identify suspected or unexpected cofactors and comorbidities. However, liver biopsy has also a number of limitations that have to be taken into account. Many recent studies clearly indicate that liver biopsy, as it is usually taken for diagnostic purpose, is prone to sampling errors and may underestimate the amount of liver fibrosis. Several studies suggest that cirrhosis might be missed on a single blind percutaneous liver biopsy in 10%-30% of cases<sup>[9-11]</sup>.

Table 1 METAVIR scoring system for liver fibrosis

Stage	Description
F0	No fibrosis
F1	Portal fibrosis without septa
F2	Portal fibrosis with few septa
F3	Septal fibrosis without cirrhosis
F4	Cirrhosis

Portal fibrosis is a stellate enlargement of portal tracts without any bridging fibrosis on the biopsy sample. Few septa mean at least one fibrous septa on the core biopsy. Theoretically, a fibrous septa is a bridge of connective tissue between two portal tracts, a portal tract and a centrolobular vein, or between two centrolobular veins. Septal fibrosis means that the liver biopsy is crossed by several septa; the transition between F2 and F3 begins when there is more fibrous septa than portal tracts without septa on the biopsy. Cirrhosis means that liver tissue is mutilated by nodular fibrosis that delineates hepatocytes nodules.

When three different liver samples were analysed, the percentage of correct diagnosis increased from 80 to 100%<sup>[12]</sup>. In more recent times, Regev and colleagues have shown that samples obtained from the right and left lobes of the liver during laparoscopy gave different fibrosis staging in one third of the cases<sup>[13]</sup>. Other studies have analysed agreement/disagreement among pathologists. Although the use of more standardised scoring systems, such as those of the Knodell's, Ishak's and METAVIR's classifications, has improved the inter-observer and intra-observer variability, there are still several factors that may significantly influence the diagnostic accuracy of a liver biopsy. The size of the liver sample is obviously very important. Colloredo *et al.* have carefully analysed the importance of the sample size for a correct stadition of liver disease in patients with chronic hepatitis C<sup>[14]</sup>. By reducing progressively the dimension of the same liver biopsy, they reported that the smaller was the sample analysed, the milder was the diagnosis made by the pathologist in relation to the stage of fibrosis. The same biopsy was diagnosed as F3-F4 when a larger part of the sample was observed and only as F1-F2 when the size of tissue was reduced in length or wideness. Other studies have reported that the type and the size of needle used is also important. The Tru-Cut needle was superior to the Menghini needle, particularly for the diagnosis of more advanced fibrosis<sup>[15-16]</sup>. A thick needle was superior to the fine needle in assessing the presence of advanced fibrosis and cirrhosis<sup>[17]</sup>. Some studies would suggest that an adequate liver biopsy sample should contain more than 5 portal tracts and be at least 15 mm in length<sup>[18-20]</sup>. However, other studies reported higher threshold for optimised accuracy. Colloredo *et al.* concluded that an adequate specimen should be at least 20 mm in length with at least 11 complete portal tracts while others have recommended even bigger samples, up to 25 mm in length<sup>[14,21]</sup>. On the same line, Scheuer has recently concluded that bigger is better<sup>[22]</sup>. The need for obtaining a liver sample of adequate size is however in contrast with the patient need of a procedure causing limited pain and hemorrhagic risks. Liver biopsy may be in fact a risky procedure for some patients, particularly for those with more advanced liver

fibrosis<sup>[23-25]</sup>. A French survey which interviewed 1177 general practitioners concluded that liver biopsy may be refused by up to 59% of patients with chronic hepatitis C and that 22% of the physicians share the same concern for this invasive procedure<sup>[26]</sup>. On this line, a recent survey assessing the consensus among Italian hepatologists on when and how to take a liver biopsy in chronic hepatitis C showed great divergence in the management of the same subgroup of patients<sup>[27]</sup>. Most recently Rousselet *et al* reported that the degree of experience of the pathologist, as indicated by longer duration of practice or belonging to an academic setting, may have an outstanding impact on the diagnostic interpretation of liver biopsy, even higher than that determined by the related to sample size<sup>[28]</sup>.

Another shortcoming of liver biopsy is its cost as it always requires hospitalisation for 6-18 hrs. A cost-benefit analysis showed that in US the cost of a liver biopsy is 1032 USD and it could raise to 2745 USD when complications occur<sup>[29]</sup>.

## **PATHOPHYSIOLOGY OF HEPATIC FIBROSIS- THE CONCEPTUAL BASIS OF NON INVASIVE BIOMARKERS**

The key step in the pathophysiology of liver fibrogenesis is the balance between extracellular matrix (ECM) deposition and removal. Indeed, the ECM metabolism is a very dynamic process, influenced by factors that contribute to its deposition and by others that mediate its degradation. The hepatic stellate cells (HSCs) are the major source of ECM. During liver injury, activation of quiescent HSCs to a proliferative, fibrogenic and contractile type of myofibroblasts is the dominant event in fibrogenesis. HSCs can be activated by several cytokines (e.g., tumor growth factor beta (TGF- $\beta$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), platelet derived growth factor (PDGF)) which are secreted in response to liver injury. On the other hand, other signals (e.g., interleukin-10 (IL-10)) promote ECM degradation. Once activated, HSCs secrete cytokines such as metalloproteinases, TGF- $\beta$ 1, PDGF, monocyte chemotaxis protein 1 (MCP-1), endothelin 1 (ET-1). Some of these are directly involved in fibrogenesis (TGF- $\beta$ 1, connective tissue growth factor), others in chemotaxis (MCP-1) and proliferation of HSCs (PDGF, ET-1) and others in matrix degradation (metalloproteinases)<sup>[30]</sup>.

The pathogenesis of liver fibrosis is somehow related to the etiology of the underlying CLD. The mechanisms by which HCV and HBV induce liver fibrosis are only partially understood. In chronic hepatitis B infection the pathogenesis of hepatic fibrosis has been associated with cytokines, particularly TGF- $\beta$ 1<sup>[31]</sup>. HCV induces oxidative stress and recruitment of inflammatory cells, with HSCs activation and collagen deposition. In addition, several HCV proteins directly stimulate the fibrogenic and flogistic pathways of HSCs<sup>[32]</sup>. A recent "in vitro" study reported that hepatitis C virus nonstructural genes (NS3 and NS5B) are able to induce increased expression of TGF- $\beta$ 1 and of other profibrogenic factors in infected hepatocytes<sup>[33]</sup>. These cellular events directly mediated by

HCV in infected hepatocytes could explain the occurrence of progressive liver fibrosis with minimal inflammation. The pathogenetic mechanisms acting in alcoholic (AFLD) and in non alcoholic fatty liver disease (NAFLD) also involve cytokine- and oxidative stress- mediated injury. In AFLD the main stimulus for cytokine release from Kupffer cells in the liver is portal endotoxemia, arising as a result of increased gut permeability caused by ethanol and its metabolism to acetaldehyde. Oxidative stress derives from ethanol metabolism, Kupffer cell activation and from the effect of TNF- $\alpha$  on hepatocyte mitochondria. The resulting activation of HSCs increases the fibrogenic and inflammatory signals<sup>[34-35]</sup>. The pathogenesis in non alcoholic steatohepatitis (NASH) is less understood and several mechanisms have been proposed and investigated. Hepatic steatosis is considered as the first of two hits in the pathogenesis of NASH, since the presence of oxidisable fat within the liver is able to trigger lipid peroxidation<sup>[36]</sup>. However, many patients with fatty liver do not progress to necroinflammation and fibrosis. The potential second hits for the development of NASH could be an increase in the expression of ethanol-inducible cytochrome P450 2E1 (CYP2E1) and in the intrahepatic concentration of free fatty acids, resulting in oxidative stress via peroxisomal oxidation<sup>[37]</sup>. It has also been hypothesised that iron, in relatively low concentrations, could synergize with lipid overload and CYP2E1 induction to trigger oxidative stress in hepatocytes<sup>[38]</sup>.

All these data and concepts are of great interest for the understanding of the various factors that contribute to progressive liver fibrosis in patients with different forms of CLD. However, their complexity and interplay clearly explain the difficulties encountered in the search of a specific and sensitive marker that could be universally valid as a diagnostic and prognostic tool to measure liver fibrogenesis in clinical practice.

## **GENETIC POLYMORPHISMS AND LIVER FIBROSIS**

The complexity of the fibrogenetic process and the high number of cytokines involved imply that several genetic polymorphisms could influence progression of liver fibrosis. To date, genetic polymorphisms linked to hepatic fibrogenesis have been investigated mainly in chronic hepatitis C and in AFLD. Table 2 describes gene polymorphisms that were reported to either favour or reduce fibrogenesis in patients with different forms of CLD. While these studies clearly indicate that many genetic factors have a definitive influence on the risk of developing a more or less active and progressive fibrogenesis, none of them have found an application as diagnostic/prognostic marker in clinical practice, due to their complexity, difficulty to test and variable behaviour in different patients populations. Better understanding of the genetic influence on liver fibrogenesis is nevertheless of paramount importance as it may lead in the near future to identification of new therapeutic targets and strategies for development of effective antifibrotic treatments.

Table 2 Gene polymorphisms described as involved in fibrogenesis

Author	Etiology of liver disease	Gene	Function of the gene product	Implicated genotype	Effect on gene product	Effect on fibrosis
Powell <sup>[39]</sup>	HCV	TGF- $\beta$ 1	Profibrogenic	Codon 25 (pro/arg)	Increased transcription	Increased
Powell <sup>[39]</sup> Forrest <sup>[40]</sup>	HCV	AT	Profibrogenic	-6 G/A	Increased transcription	Discordant results
Bonkovsky <sup>[41]</sup> Negro <sup>[42]</sup> Chitturi <sup>[43]</sup> Geier <sup>[44]</sup>	HCV / NASH	HFE	Iron metabolism	C282Y, H63D	Iron overload	Discordant results
Yee <sup>[45]</sup> Grove <sup>[46]</sup>	HCV / AFLD	TNF- $\beta$	Proinflammatory	-308 G/A	Increased transcription	Increased
Grove <sup>[47]</sup> Knapp <sup>[48]</sup>	HCV / AFLD	IL-10	Immune modulation	-1082 A/A; -627 C/A	Decreased transcription	Increased
Reynolds <sup>[49]</sup>	HCV	MPO	Bactericide	-463 G/A	Increased transcription	Increased
Wozniak <sup>[50]</sup>	HCV	ApoE	Viral entry to cells	E4 allele	Abnormal function	Reduced
Muhlbauer <sup>[51]</sup>	HCV	MCP-1	Proinflammatory	-2518 G/A	Increased transcription	Increased
Wright <sup>[52]</sup>	HCV	Factor V Leiden	Thrombin generation	Codon 560 (arg/gln)	Resistance to activation	Increased
Romero-Gomez <sup>[53]</sup>	HCV	SLC11A1	Macrophage function	Homozygosity (GT) <sub>5</sub> AC(GT) <sub>10</sub> G (Allele 2) in the promoter region	Poor promoter	Reduced
Adinolfi <sup>[54]</sup>	HCV	MTHFR	Folate metabolism	C677T	HyperHC	Increased
Okamoto <sup>[55]</sup>	HCV	MMP	Matrix degradation	1G/2G (MMP-1), 5A/6A (MMP-3), C/T (MMP-9)	Reduced transcription	More frequent in cirrhosis than CHC
Yamauchi <sup>[56]</sup> Okamoto <sup>[57]</sup> Frenzer <sup>[58]</sup>	AFLD	CYP2E1	Ethanol metabolism	c1/c1, c2/c2 alleles	Increased activity	Discordant results
Yamauchi <sup>[56]</sup> Frenzer <sup>[58]</sup>	AFLD	ADH	Ethanol metabolism	ADH2 c1,c2,c3 alleles; ADH3 c1, c2, c3 alleles	Abnormal function	Discordant results
Yamauchi <sup>[56]</sup> Okamoto <sup>[57]</sup>	AFLD	ALDH	Ethanol metabolism	ALDH2 c2/c2 alleles	Abnormal function	Discordant results
Burim <sup>[59]</sup>	AFLD	CYP1A1	Ethanol metabolism	m2/m2 allele	Increased activity	Increased
Dixon <sup>[60]</sup>	NAFLD	AT and TGF- $\beta$	Profibrogenic	High AT and TGF- $\beta$ 1 producing polymorphisms	Increased activity	Increased

TGF- $\beta$  = transforming factor beta; AT = angiotensinogen; HFE = hereditary haemochromatosis gene; NASH = non alcoholic steatohepatitis; TNF- $\beta$  = tumor necrosis factor beta; AFLD = alcoholic liver disease; IL-10 = interleukin 10; NAFLD = non alcoholic fatty liver disease; MPO = myeloperoxidase; ApoE = apolipoprotein E; MCP-1 = monocyte chemotactic protein 1; SLC11A1 = solute carrier family 11 member 1; MTHFR = methylenetetrahydrofolate reductase; HC = homocysteinemia; MMP = metalloproteinase; CYP2E1 = cytochrome P 2E1; ADH = alcohol dehydrogenase; ALDH = aldehyde dehydrogenase; CYP1A1 = cytochrome P A1.

## SEARCHING FOR THE IDEAL NON INVASIVE MARKER OF LIVER FIBROSIS: THE HOLY GRAIL OF THE CLINICAL HEPATOLOGIST

In the last decade, many studies have been dedicated to the search of non invasive markers able to provide an accurate information about liver fibrogenesis activity and fibrosis stage in patients with chronic, potentially progressive, hepatic diseases. The ideal characteristics of such a marker are summarised in Table 3. Two main, quite different, approaches have been followed. Many studies have been dedicated to the evaluation of "direct" markers of fibrogenesis, i.e. of biochemical parameters, measurable in the peripheral blood as direct expression of either the deposition or the removal of ECM in the liver. These direct markers of liver fibrosis include several glycoproteins (hyaluronan, laminin, human cartilage glycoprotein 39 (YKL-40)), the collagens family (procollagen III, type IV collagen and type IV collagen 7s domain), the collagenases and their inhibitors (metalloproteinases and tissue inhibitors of metalloproteinases) and a number

Table 3 Features of the ideal marker of liver fibrosis

Specific for fibrosis of the liver
Providing measurement of: A) stage of fibrosis, B) fibrogenesis activity
Not influenced by comorbidities (e.g. renal, reticulo-endothelial)
Known half-life
Known excretion route
Sensitive
Reproducible

of cytokines connected with the fibrogenetic process (TGF- $\beta$ 1, TNF- $\beta$ ). These markers and their role in fibrogenesis are described in Table 4. The potential clinical applications of such markers appear extremely interesting and innovative, as they could be used not only to stage liver fibrosis, but also and more appropriately to assess the speed of liver fibrogenesis with most relevant prognostic value, and also to estimate and monitor the efficacy of and the response to antifibrotic drugs. However, these ambitious goals have not been yet achieved and the described direct makers of fibrogenesis have been so far tested only for their performance in defining the actual



Table 4 Direct non invasive markers of liver fibrosis

Author	Liver disease	Marker	Characteristics	Pathophysiology
McHutchison <sup>[61]</sup> Murawaki <sup>[62]</sup> Halfon <sup>[63]</sup> Pares <sup>[64]</sup> Suzuki <sup>[65]</sup> Naveau <sup>[66]</sup> Santos <sup>[67]</sup> Montazeri <sup>[68]</sup>	HCV, AFLD, NAFLD, HBV	Hyaluronic acid	Component of the ECM in every tissue	Synthesized by HSCs and degraded by sinusoidal endothelial cells
Santos <sup>[67]</sup> Walsh <sup>[69]</sup>	HCV, NAFLD	Laminin	Co-expressed with type IV collagen in basement membranes	Increased deposition in viral disease
Tran <sup>[70]</sup> Saitou <sup>[71]</sup>	AFLD, HCV	YKL-40	Human cartilage glycoprotein	Involved in remodelling and degradation of ECM
Sakugawa <sup>[72]</sup> Murawaki <sup>[73]</sup> Walsh <sup>[69]</sup> Saitou <sup>[71]</sup> Santos <sup>[67]</sup>	HCV, NAFLD	Type IV collagen/ 7s domain	Main collagen component of the basement membrane	Increase with severity of liver fibrosis
Guechol <sup>[74]</sup> Pares <sup>[64]</sup>	HCV, AFLD	Procollagen III	Propeptide	Released during matrix deposition and remodelling
Boeker <sup>[75]</sup> Murawaki <sup>[62]</sup>	HCV	MMP-2	Collagenase	Correlates with fibrosis
Boeker <sup>[75]</sup>	HCV	TIMP-1	Metalloproteinase	Inhibitor of collagenase
Patel <sup>[76]</sup>	HCV	Three-marker panel	Combination of hyaluronic acid, TIMP-1, $\alpha_2$ M	Better accuracy by combined markers

AFLD = alcoholic fatty liver disease; NAFLD = non alcoholic fatty liver disease; ECM = extracellular matrix; HSCs = hepatic stellate cells; YKL-40 = human cartilage glycoprotein-39; MMP-2 = metalloproteinase 2; TIMP-1 = tissue inhibitor metalloproteinase 1;  $\alpha_2$ M = alfa-2-macroglobulin.

stage of liver fibrosis, with variable results (Tables 6 and Table 7). A second and easier approach in the search of non invasive markers of liver fibrosis has been to take single or combined haematological or biochemical parameters that reflect the stage of liver disease and to assess and compare the accuracy of their diagnostic performance. This approach, that often uses routinely performed blood tests, has led to the identification of sets of markers able to define the stage of liver fibrosis with an accuracy very similar, if not superior, to that of the more sophisticated and difficult to test direct markers. The diagnostic performance of most direct and indirect markers of liver fibrosis has been investigated in all the common etiological forms of CLDs, including hepatitis C, hepatitis B and alcoholic and non alcoholic fatty liver and steatohepatitis, although some of them have been more extensively tested in patients with chronic hepatitis C.

## WHAT THE IDEAL NON INVASIVE MARKER OF LIVER FIBROSIS SHOULD IDENTIFY?

Most non invasive markers of liver fibrosis described in the literature were developed with the aim of discriminating between "insignificant" (F0-F1 by METAVIR) and clinically "significant" fibrosis ( $\geq$  F2 by METAVIR) or of identifying or excluding established cirrhosis in patients with well compensated CLD. Both these aims are clinically most relevant. Presence of significant fibrosis in the liver is indeed considered as the hallmark of a progressive liver disease and a clear indication for immediate initiation of antiviral therapy in patients with chronic HCV or HBV infection, in agreement with International and National Guidelines and recommendations for the management of these conditions<sup>[1,77-78]</sup>. On the other hand, patients

with F0-F1 usually do not progress or progress much slowly<sup>[79-80]</sup>. Presence of cirrhosis, even when fully compensated and still clinically occult, indicates the need for specific monitoring of complications related to portal hypertension and to the increased risk of developing hepatocellular carcinoma. Furthermore, patients with HCV or HBV related cirrhosis are less likely to respond to interferon-based antiviral therapy and at higher risk of hepatic decompensation in the case of significant ALT flares when relapsing after therapy withdrawal.

## THE DIRECT MARKERS OF LIVER FIBROGENESIS

A list of direct markers of liver fibrogenesis are described in Table 4 while Table 6 and 7 report their diagnostic performance in detecting significant fibrosis ( $\geq$  F2 by METAVIR) and cirrhosis, respectively, in the different etiologic forms of CLDs in which they have been evaluated. Hyaluronic acid has been extensively studied in hepatitis C and AFLD and, in more recent years, it has also been tested in smaller cohorts of patients with NAFLD and hepatitis B. Overall, a rather good accuracy of hyaluronic acid in the different studies in discriminating significant from insignificant fibrosis has been reported, with an area under the curve (AUC) ranging from a minimum of 0.78 in NAFLD to an excellent 0.98 in hepatitis B (Table 6). However, the number of patients tested was quite low in both these patients categories, with 75 and 112 cases in two studies on NAFLD (AUC of 0.87 and 0.79, respectively)<sup>[65,72]</sup> and only 65 patients in the single study conducted in chronic hepatitis B<sup>[68]</sup>. Further studies are clearly needed, especially in hepatitis B since the accuracy reported by Montazeri *et al* was excellent (0.98 AUC) but should now be confirmed in larger studies. In chronic hepatitis C, the ability of hyaluronic

acid to differentiate minimal-mild from moderate-severe fibrosis has been tested in much larger series of patients. In various cohort studies the AUC values have ranged from 0.82 to 0.92 (Table 6). In a study conducted in 326 patients the AUC was 0.86 and the specificity was 95% for significant fibrosis while the AUC was 0.92 and the specificity was 89.4% for cirrhosis when a cut off level of 110  $\mu\text{g/L}$  was used<sup>[74]</sup>. However, another cohort study with more than 400 cases has reported an AUC of only 0.73 for significant fibrosis<sup>[63]</sup>. In the same study, cirrhosis could be excluded with excellent negative predictive value and sensitivity (100%) using a cut off level of 50  $\mu\text{g/L}$ . Similar results were reported in another study of 486 patients in which hyaluronic acid levels < 60  $\mu\text{g/L}$  excluded cirrhosis with 99% negative predictive value<sup>[61]</sup>. In a smaller study hyaluronic acid performed less well in excluding cirrhosis, with an AUC of 0.85 and 80% negative predictive value<sup>[71]</sup>. In AFLD the performance of hyaluronic acid for significant fibrosis varied significantly<sup>[64,66]</sup> while the marker showed very good performance for cirrhosis, with an AUC of 0.93<sup>[66]</sup>. On the basis of these findings, the greatest clinical utility of hyaluronic acid might be in its ability in excluding cirrhosis. The results of a study conducted in 79 patients with NAFLD were also encouraging, as hyaluronic acid had a 0.92 AUC value for cirrhosis<sup>[65]</sup>. Further studies with larger series of cases are needed, especially in NAFLD and chronic hepatitis B.

Among the glycoproteins, laminin has been assessed as a non invasive marker mainly for significant liver fibrosis. It showed an overall accuracy of 81% in a detailed study on 243 patients with CLDs<sup>[81]</sup>. It performed better in AFLD (84% accuracy) than in viral hepatitis (77% accuracy). Another study of 37 patients with chronic hepatitis C showed slightly better performance (AUC = 0.82)<sup>[69]</sup>. In a recent study conducted on 30 patients with NAFLD laminin showed good performance, particularly when combined with type IV collagen, with 87% accuracy, 100% specificity and positive predictive value<sup>[67]</sup>.

YKL-40 is a recently described glycoprotein that belongs to the chitinase family. It is strongly expressed in human cartilage and human liver. YKL-40 is a relatively new marker of hepatic fibrosis and it has been only preliminarily evaluated in CLDs. It was initially investigated in patients with alcoholic liver disease. In a cohort of 146 heavy drinkers, YKL-40 showed good specificity (88.5%) but poor sensitivity (50.8%)<sup>[70]</sup>. Better results have been reported in 109 patients with chronic hepatitis C, with 0.81 AUC, 78% sensitivity and 81% specificity<sup>[71]</sup>. In the same study the accuracy in predicting cirrhosis was however lower, with an AUC of 0.795. Further studies are needed to elucidate the value of this new non invasive marker of liver fibrosis in chronic viral hepatitis and in fatty liver diseases.

Among the collagens, type IV collagen has been extensively investigated as non invasive marker of liver fibrosis. Type IV collagen is composed of a major triple-helix, an amino-terminal triple-helix (7s domain) and a carboxy-terminal globular domain. The first two forms of type IV collagen have been used in clinical studies. Type IV collagen has been studied in hepatitis C and NAFLD and a good diagnostic performance for significant fibrosis

has been reported, particularly in hepatitis C (AUC = 0.83)<sup>[69,73]</sup>. Murawaki *et al* have focused on the 7s domain and central triple helix domain and found a slightly better accuracy of the former in detecting cirrhosis, with 75% positive predictive value and 92% negative predictive value<sup>[73]</sup>. The role of 7s domain has also been investigated in 112 patients with NAFLD and its performance has been compared with hyaluronic acid<sup>[72]</sup>. The results showed a better diagnostic accuracy for type IV collagen-7s domain (0.828 *vs* 0.797 AUC, respectively). Several studies have also compared the diagnostic performance of type IV collagen with that of hyaluronic acid in hepatitis C and reported the superiority of the latter marker<sup>[62,71]</sup>. These findings would indicate no definitive advantage in using type IV collagen instead of hyaluronic acid in hepatitis C. Data on type IV collagen in NAFLD are extremely limited and need further evaluation.

Several studies evaluated a possible role of procollagen III in hepatitis C and in AFLD. In comparative studies conducted in hepatitis C, procollagen III performed less well than type IV collagen and hyaluronic acid<sup>[71,74]</sup>. On the other hand, a good accuracy has been described in AFLD (AUC = 0.867), but again it was slightly worse than hyaluronic acid (0.913)<sup>[64]</sup>. The superiority of hyaluronic acid and type IV collagen does not allow to recommend the use of procollagen III as non invasive marker of liver fibrosis.

Collagenases and their inhibitors have also been proposed as surrogate markers of liver fibrosis. Those reported to have some clinical impact include metalloproteinase 2 (MMP-2) and tissue inhibitor metalloproteinase 1 (TIMP-1)<sup>[82]</sup>. In a recent study Boecker and colleagues investigated the role of MMP-2 and TIMP-1 as non invasive markers of liver fibrosis in 78 patients with chronic hepatitis C<sup>[75]</sup>. Both these proteins were measured with two different methods and their diagnostic performance was different. However, with both methods, the performance in detecting cirrhosis was very high, especially for MMP-2 (0.97 AUC). Unfortunately, it has been difficult to obtain good standardisation of the method for routine clinical use.

Measurements of serum cytokines (TGF- $\beta$ , TNF- $\beta$ ) involved in fibrogenesis have been assessed in a limited number of studies in which cytokines were found to have somehow less value in predicting liver fibrosis compared to the ECM tests<sup>[20,83]</sup>. Several Authors have tried to combine different direct markers of liver fibrosis. In a cohort study of more than one thousand patients with CLD an algorithm combining hyaluronic acid, procollagen III and TIMP-1 has been described<sup>[84]</sup>. The AUC was discrete for hepatitis C (0.77), good in NAFLD (0.87) and excellent in AFLD (0.94). Another combination panel of matrix markers (hyaluronic acid, TIMP-1 and  $\alpha_2$ -macroglobulin) has been tested in a cohort of HCV patients, obtaining an AUC of 0.83 with an accuracy of 75%<sup>[76]</sup>. The diagnostic performance of this combination panel appears quite similar to those reported for some single ECM components, particularly hyaluronic acid, laminin and YKL-40. Santos *et al* have recently investigated some ECM components in NAFLD showing that a combination of laminin and type IV collagen (282 ng/mL and 145 ng/mL

Table 5 Indirect non invasive markers of liver fibrosis

Authors	Liver disease	Biomarker	Description	Rationale
Giannini <sup>[85]</sup>	HCV, NAFLD	AAR	AST to ALT ratio	AST and ALT levels increase with progressive fibro-
Wai <sup>[86]</sup> Macias <sup>[87]</sup>	HCV, HIV/HCV	APRI	AST to platelet ratio	Statistical association with liver fibrosis
Forns <sup>[88]</sup> Macias <sup>[87]</sup>	HCV, HIV/HCV	Forns' index	Combination of age, platelet, $\gamma$ GT, cholesterol	Statistical association with liver fibrosis
Islam <sup>[89]</sup>	HCV	GUCI	Combination of AST, INR, platelet	Statistical association with liver fibrosis
Imbert-Bismut <sup>[90]</sup> Myers <sup>[91]</sup>	HCV, HIV/HCV,	Fibrotest	Combination of $\alpha$ 2M, ApoA1,	Statistical association with liver fibrosis
Myers <sup>[92]</sup> Naveau <sup>[66]</sup>	HBV, AFLD		bilirubin, $\gamma$ GT, haptoglobin	
Sud <sup>[93]</sup>	HCV	FPI	Combination of HOMA-IR, age, cholesterol, AST, alcohol intake	Statistical association with liver fibrosis
Callewaert <sup>[94]</sup>	CLDs (mostly HCV)	Glycoccirrho test	Profiles of serum protein N-glycans	Glycoproteins are produced mainly by hepatocytes

NAFLD = non alcoholic fatty liver disease; AAR = aspartate to alanine aminotransferase ratio;  $\alpha$ 2M = alfa-2-macroglobulin; ApoA1 = apolipoprotein A1; APRI = AST to platelet ratio index; GUCI = Goteborg University Cirrhosis Index; INR = international normalised ratio; FPI = fibrosis probability index; HOMA-IR = homeostasis model assessment of insulin resistance; CLDs = chronic liver diseases.  $\gamma$ GT = gamma glutamil transpeptidase.

cuts off, respectively) could individuate patients with significant fibrosis with 100% positive predictive value and specificity<sup>[67]</sup>. Unfortunately, only 30 patients were included in this study. In conclusion, combination panel of ECM components may improve the diagnostic accuracy of the single markers, particularly in AFLD and NAFLD, while no clear advantage has been so far demonstrated in patients with chronic hepatitis C. Further studies are needed to validate the new combinations of markers recently proposed for AFLD and NAFLD.

## THE INDIRECT MARKERS OF LIVER FIBROSIS

One of the main limitation to the clinical use of direct markers of liver fibrosis is that they are not routinely available in all hospital settings. While direct markers of liver fibrosis reflect the process of fibrogenesis, indirect markers satisfy the request for a simple and easy to perform marker. The indirect markers of liver fibrosis are described in Table 5. Their diagnostic performance in detecting significant fibrosis and cirrhosis is reported in Table 6 and Table 7, respectively. The first indirect marker of liver fibrosis were transaminases, later associated in the aspartate to alanine aminotransferase ratio (AAR) to detect cirrhosis<sup>[85]</sup>. The strength of such marker is the simplicity and the immediate availability for every Hepatologist and Clinician. On the other side, the numerous studies conducted showed that its accuracy is highly variable<sup>[83]</sup>. Moreover, it cannot be used to differentiate between no-mild and moderate-severe fibrosis. A further evolution of this index was later introduced by Wai *et al* who combined aspartate aminotransferase (AST) with platelet count<sup>[86]</sup>. This AST to platelet ratio index (APRI) was then assessed in several studies conducted with a cohort of patients with hepatitis C and showed a rather good diagnostic performance and reproducibility, particularly for cirrhosis (AUC range from 0.77 to 0.94)<sup>[86,95-97]</sup>. The real strength of such index is that it is based on blood tests that are routinely performed in patients with liver disease with no need for additional blood collection

Table 6 Diagnostic performance of non invasive markers of liver fibrosis in discriminating between no-mild fibrosis (F0-F1 by METAVIR) and moderate-advanced fibrosis (F  $\geq$  2 by METAVIR)

Marker	Disease	Sensitivity	Specificity	AUC	References
<b>Direct markers of liver fibrosis</b>					
Hyaluronic acid	HCV	75-79	80-100	0.82-0.92	[61-62]
	HBV	91	98.1	0.98	[68]
	AFLD	87	93	0.79-0.91	[64,66]
	NAFLD	66-85	68-91	0.78-0.87	[65,67]
Laminin	HCV	80	83	0.82	[72]
	NAFLD	82	89	n.a.	[69,81]
YKL-40	AFLD	88.5	50.8	n.a.	[67]
	HCV	78	81	0.81	[70]
Type IV collagen	HCV	73-80	81-85	0.83	[71]
	NAFLD	64	89	n.a.	[69,73]
Type IV collagen-7s	HCV	74-83	75-88	n.a.	[67]
	NAFLD	70	81	0.83	[73]
Procollagen III	HCV	60-78	74-75	0.69	[72]
	AFLD	80	87	0.87	[71,74]
MMP-2	HCV	7-75	70-100	0.59	[64]
TIMP-1	HCV	67	68	0.71	[62,75]
Three marker panel	HCV	77	73	0.83	[75]
<b>Indirect markers of liver fibrosis</b>					
APRI	HCV	41-91	47-95	0.69-0.88	[86,95-97]
	HIV/HCV	51	91	0.8	[87]
Forns' index	HCV	79.8-94	95-98.3	0.78-0.86	[88,98]
	HIV/HCV	43	96	0.77	[89,97]
Fibrotest	HCV	65-87	59-80.6	0.74-0.87	[87]
	HIV/HCV	90	60	0.85	[90,97]
	HBV	34	93	0.78	[91]
	AFLD	88	60	0.84	[92]
FPI	HCV	85-96	94-98	0.77	[66]
					[93]

AUC=area under the curve; AFLD=alcoholic fatty liver disease; NAFLD=non-alcoholic fatty liver disease; n.a.=not available; YKL-40=human cartilage glycoprotein-39; MMP-2=metalloproteinase 2; TIMP-1=tissue inhibitor metalloproteinase 1; APRI=AST to platelet ratio index; FPI= fibrosis probability index.

or costs. Most recently, APRI has been modified by adding alanine aminotransferase (ALT) and international normalised ratio (INR), with further improvement of the diagnostic accuracy, particularly for cirrhosis<sup>[103]</sup>. Another rather simple index has been described by Forns *et al*<sup>[88]</sup>. It derives from combination of age, cholesterol level, gamma glutamyl transpeptidase ( $\gamma$ GT) and platelet count and was developed to differentiate no/minimal (F0-F1) from significant ( $\geq$  F2) fibrosis in hepatitis C, while it gives no informations on cirrhosis. It has been suggested that Forns' index might be less accurate in patients with HCV genotype 3 that is associated with very low cholesterol levels<sup>[99]</sup>. A recent study by Macias and colleagues have tested the role of APRI and Forns' index as non invasive markers of liver fibrosis in 357 patients coinfecting with human immunodeficiency virus (HIV) and HCV<sup>[87]</sup>. Overall, the markers showed a lower diagnostic accuracy than in HCV mono-infected patients. The most important limits of both APRI and Forns' index are in the fact that they leave almost half of the patients unclassified.

Another index that combines together standard biochemical serum markers such as AST, platelet count and INR has been reported<sup>[89]</sup>. It showed good accuracy for cirrhosis in hepatitis C, but without significant improvement with respect to the individual tests used alone. The most widely investigated combination set of non invasive markers of liver fibrosis is Fibrotest. This was initially proposed in 1999 by Imbert-Bismut and colleagues as fibroscore and uses a combination of five blood tests including  $\gamma$ GT, bilirubin, haptoglobin, apolipoprotein A1,  $\alpha$ 2-macroglobulin, adjusted for gender and age<sup>[90]</sup>. Fibrotest and Fibrosure are the commercially available equivalents of fibroscore in Europe and in the US, respectively. Fibrotest-Fibrosure has the great advantage of classifying all stages of liver fibrosis and it does not leave any patient unclassified. On the other hand, it uses two rather uncommon parameters, apolipoprotein A1 and  $\alpha$ 2-macroglobulin and requires precise standardisation of the laboratory procedures<sup>[104]</sup>. To date, Fibrotest-Fibrosure is by far the most investigated and validated non invasive marker of liver fibrosis with around 20 studies reported in the literature. It has been extensively tested in chronic hepatitis C where it shows an AUC of around 0.85 for significant fibrosis. Fibrotest was also tested in chronic hepatitis B and it performed slightly worse, with an AUC of 0.78 in the only study conducted<sup>[92]</sup>. In the only study performed in HIV/HCV coinfecting patients Fibrotest performed well, particularly for cirrhosis (AUC = 0.87) that could be excluded with 100% negative predictive value<sup>[91]</sup>. The same performance on AFLD was obtained in another study from Poynard's group and the accuracy for cirrhosis was particularly high, with an AUC of 0.95<sup>[66]</sup>. However, no validation study of Fibrotest-Fibrosure has been conducted in HIV/HCV coinfection, chronic HBV infection and AFLD. A recent comparative study of indirect markers of liver fibrosis conducted in our Unit on 190 patients with chronic hepatitis C tested the performance of APRI, Forns and fibrotest<sup>[97]</sup>. Fibrotest showed the best accuracy, with an AUC of 0.81 for significant fibrosis and of 0.71 for cirrhosis. The same study was also one of the few reporting data on non invasive markers of liver

**Table 7** Diagnostic performance of non invasive markers of liver fibrosis in detecting cirrhosis

Marker	Disease	Sensitivity	Specificity	AUC	References
<b>Direct markers of liver fibrosis</b>					
Hyaluronic acid	HCV	80-100	79-89.4	0.85-0.92	[61,63] [71,74]
	AFLD	99	80	0.93	[66]
	NAFLD	n.a.	n.a.	0.92	[65]
YKL-40	HCV	80	71	0.79	[71]
Type IV collagen	HCV	60	61	n.a.	[71,73]
Procollagen III	HCV	60-77	66-74	0.73	[71,74]
MMP-2	HCV	74-83	96-100	0.97	[75]
TIMP-1	HCV	100	56-75	0.9	[75]
<b>Indirect markers of liver fibrosis</b>					
AAR	HCV	47-81.3	55.3-97		[85,102] [96]
APRI	HIV/HCV	38	77	0.6	[87]
	HCV	38.4-57	86.7-93	0.61-0.94	[86,95-97]
	HIV/HCV	53	89	0.79	[87]
GUCI	HCV	80	78	0.85	[89]
Fibrotest	HCV	13-50	91-98	0.71-0.87	[90,97] [100,101]
	HIV/HCV	100	65	0.87	[91]
	HBV	18	99	0.78	[92]
	AFLD	99	83	0.95	[66]
	Glycocirrho test	79	86	0.87	[94]

AUC = area under the curve; AFLD = alcoholic fatty liver disease; NAFLD = non-alcoholic fatty liver disease; YKL-40 = human cartilage glycoprotein-39; MMP-2 = metalloproteinase 2; TIMP-1 = tissue inhibitor metalloproteinase 1; AAR = aspartate to alanine aminotransferase ratio; APRI = AST to platelet ratio index; GUCI = Goteborg University Cirrhosis Index.

fibrosis in patients with persistently normal transaminases (PNALT). The three non invasive markers of liver fibrosis were tested in 65 HCV patients with PNALT and APRI showed the best accuracy, with an AUC of 0.77 (Table 8). Available data would suggest that Fibrotest-Fibrosure performs well in detecting the two extremes of the staging range of liver fibrosis (F0-1 and F4), while it might performs somehow less well in the intermediate stage (F2)<sup>[20,101]</sup>. In some validation studies Fibrotest-Fibrosure was not so accurate as described in the early studies<sup>[100]</sup>. Fibrotest-Fibrosure has also been evaluated in combination with other markers. Callewaert and colleagues have recently proposed a non invasive marker based on profiles of serum glycoproteins (Glycocirrhohet)<sup>[94]</sup>. They found that the combination of Fibrotest-Fibrosure and Glycocirrhohet allows identification of cirrhosis with 100% specificity and 75% sensitivity. Another study has recently considered the influence of metabolic factors in the development of fibrosis in hepatitis C and proposed an index that includes assessment of insulin resistance and alcohol consumption. The performance of this approach was however inferior to that of simpler markers (0.77 of AUC) and has not been yet externally validated<sup>[93]</sup>. Recently a new technology (Fibroscan) that measures liver stiffness has been proposed<sup>[105]</sup>. The rationale is based on the



**Table 8** Diagnostic performance of APRI, Forns' index, Fibrotest and of sequential algorithms combining the three markers in patients with chronic hepatitis C

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	AUC	Classified patients(%)
<b>Detection of significant fibrosis in chronic hepatitis C with elevated ALT</b>							
APRI	29.7	93.8	95.7	52.7	60.2	0.69	54.1
Fibrotest	65	80.6	80	66.7	72.6	0.81	100
Forns	24.3	98.3	94.7	50.9	57.1	0.79	55.5
Sequential algorithm	100	83.8	92.7	100	94.2	n.a.	100
<b>Detection of significant fibrosis in chronic hepatitis C with PNALT</b>							
APRI	26.9	100	100	56.8	62.7	0.77	74
Fibrotest	58.3	91.3	77.7	80.7	80	0.71	100
Forns	11.5	100	100	52.1	54.9	0.58	56
Sequential algorithm	100	87.5	94.3	100	96.3	n.a.	100
<b>Detection of cirrhosis in chronic hepatitis C</b>							
APRI	38.4	86.7	38.5	86.7	78.1	0.61	54.1
Fibrotest	50	92.9	57.9	90.5	85.9	0.71	100
Sequential algorithm	94.6	95.1	78.3	99.1	95.5	n.a.	100

PPV = positive predictive value; NPV = negative predictive value; AUC = area under the curve; APRI = aspartate aminotransferase to platelets ratio; Forns = Forns' index; n.a. = not available; PNALT = persistently normal ALT.

correlation between liver fibrosis and stiffness. This new method of measuring liver fibrosis has been evaluated in a prospective study conducted in more than seven hundred patients with CLDs of different aetiologies obtaining an AUC ranging between 0.8 for detecting significant fibrosis to 0.96 for diagnosing cirrhosis<sup>[106-107]</sup>. Fibroscan has also been combined with Fibrotest-Fibrosure in a series of patients with hepatitis C obtaining an AUC of 0.88<sup>[108]</sup>. The limitation of this new technology is that it requires a costly device to measure liver stiffness, on the other hand it showed great accuracy for cirrhosis. A detailed description of the several studies recently published on the use of Fibroscan in assessing fibrosis in patients with CLDs is not within the purposes of this review aimed to discuss non invasive diagnosis of liver fibrosis by biochemical markers and blood tests.

## CURRENT USE OF NON INVASIVE MARKERS OF LIVER FIBROSIS IN CLINICAL PRACTICE AND FUTURE PROSPECTIVES

Although several non invasive markers of liver fibrosis have been developed in the last decade, their implementation in clinical practice has been slow and is still limited. According to many experts in the field, both liver pathologists and clinical hepatologists and also to the most recent international guidelines and recommendations, inter-laboratory variability, lack of reproducibility and, most importantly, an expected rate of misdiagnosis of at least 20% do not yet allow to recommend the use of most of these methods in substitution of liver biopsy<sup>[20,78,109]</sup>. One of the major limitations may be in the lack of reliable identification and classification of the intermediate stages of fibrosis<sup>[83]</sup>. On the other hand, from a clinical and not merely statistical point of view what the hepatologist needs is a diagnostic tool that, being on one side non invasive and therefore much more acceptable by the patient compared

to liver biopsy, should however ensure not to misdiagnose fibrosis and particularly not to underestimate the stage of fibrosis and presence of cirrhosis. With currently available non invasive tests, this goal cannot be achieved in all patients. The most rational way of using them is therefore that of a compromise in which non invasive markers are first used to classify those patients in which they perform with high accuracy, limiting liver biopsy to the subset in which precise non invasive staging is not possible. Obviously, the indication to take or not to take liver biopsy in the cases in which non invasive markers perform less well will also depend on the need of obtaining a more or less accurate definition of the exact stage of fibrosis and will therefore vary according to the patient's characteristics. As an example, in the elderly population with chronic HBV or HCV infection or fatty liver distinction between minimal and advanced fibrosis may be sufficient for clinical decision independently of obtaining a more precise semiquantitative staging of fibrosis. On the other hand, more precise staging may be required in other patients categories such as those with chronic HBV or HCV infection who are candidate for antiviral treatment, since the decision to start therapy and also the type of drugs to be used may be influenced by fibrosis stage.

The use in clinical practice of non invasive markers of liver fibrosis will most likely increase in the near future as they become more validated and the indication for their selective use in specific patients categories is better clarified and standardised.

## SEQUENTIAL ALGORITHMS OF NON-INVASIVE MARKERS OF LIVER FIBROSIS REDUCE THE NEED FOR LIVER BIOPSY IN HEPATITIS C

Recently we have proposed new combination algorithms of non invasive markers for assessing liver fibrosis in

chronic hepatitis C<sup>[97]</sup>. This represents the first application of a panel of markers used sequentially. Three different algorithms were developed by combining APRI, Forns' index and Fibrotest (Table 8). The rationale was that each individual test has advantages and limitations. APRI and Forns' index leave many patients unclassified while Fibrotest is more expensive and uses two uncommon parameters. Furthermore, the diagnostic accuracy of these methods does not exceed 80%-85% when they are used individually. In the first algorithm, significant fibrosis ( $\geq$  F2 by METAVIR) was identified in patients with elevated transaminases with high diagnostic performance ( $> 94\%$  accuracy) using APRI as screening test, followed by Fibrotest in APRI non-classified cases and restricting liver biopsy to patients classified F0-F1 by non-invasive tests. In the second algorithm excellent accuracy (95%) in identifying cirrhosis was achieved using a similar algorithm with different cut-off levels, limiting by 60%-70% the need of liver biopsy. This marked reduction in the need of taking a liver biopsy and the fact that our algorithm restrict this invasive procedure to patients with low chance of having cirrhosis (being classified as F0-F1 by non-invasive markers) is particularly important since the risk of liver biopsy complications is increased in the presence of cirrhosis. We have also developed an algorithm for identifying patients with significant fibrosis among HCV carriers with PNALT. This category has not been considered in most previous studies of non-invasive markers of fibrosis. However, there is abundant evidence in the literature that around 15%-30% of them may have significant fibrosis and a definitive indication to antiviral therapy<sup>[110]</sup>, particularly when considering the favourable results recently reported in such patients with PEG-interferon alfa-2a plus ribavirin combination therapy<sup>[111]</sup>. The algorithm we have developed in this specific subset of patients reduces by 50% the number of liver biopsies, and shows 93%-95% accuracy in detecting or excluding significant liver fibrosis. However, this subgroup of patients remains difficult-to-diagnose with non-invasive markers and liver biopsy is still necessary in around 50% of the cases.

## CONCLUSION

Many biomarkers of liver fibrosis have been recently proposed with the aim of substituting liver biopsy. The evidences of the literature on these markers are consistent in showing that: (1) the direct markers of liver fibrosis may have a value in excluding cirrhosis, particularly in hepatitis C (hyaluronic acid, MMP-2), and in predicting fibrosis in NAFLD and hepatitis B (hyaluronic acid, type collagen IV) but further studies are needed especially in the last two patients subgroups; (2) the indirect markers of liver fibrosis may have a value in excluding HCV-related cirrhosis (APRI, Fibrotest), HCV-related cirrhosis with HIV coinfection and cirrhosis related to AFLD (Fibrotest) but further studies are needed in large cohorts of patients; (3) combination panels of non-invasive biomarkers may improve the accuracy of the single tests (algorithm combining hyaluronic acid, procollagen III and TIMP-1 in patients with AFLD, combination of laminin

and type IV collagen in NAFLD) but further external validation is needed; (4) a series of algorithms based on sequential combination of non-invasive biomarkers have shown high diagnostic accuracy in identifying significant fibrosis and cirrhosis in patients with chronic hepatitis C with a reduction by more than 50% in the need of taking liver biopsies; (5) based on these findings, it is conceivable to anticipate that non-invasive markers of fibrosis will become in the near future an important tool in clinical practice; however, implementation of these tests in the diagnostic management of CLDs is expected to reduce but not completely abolish the need for liver biopsy; (6) for future research, priority should be given to large scale validation studies of the most promising non-invasive markers and of their combinations in the different forms of CLDs accompanied by progressive fibrosis. There is also an urgent need for better assessment and validation of direct fibrogenesis markers that could be implemented in the prognostic evaluation and in dynamic testing of the efficacy of antifibrotic interventions and treatments.

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