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Chronic hepatitis C and liver fibrosis

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Abstract

Chronic infection with hepatitis C virus (HCV) is a leading cause of liver-related morbidity and mortality worldwide and predisposes to liver fibrosis and end-stage liver complications. Liver fibrosis is the excessive accumulation of extracellular matrix proteins, including collagen, and is considered as a wound healing response to chronic liver injury. Its staging is critical for the management and prognosis of chronic hepatitis C (CHC) patients, whose number is expected to rise over the next decades, posing a major health care challenge. This review provides a brief update on HCV epidemiology, summarizes basic mechanistic concepts of HCV-dependent liver fibrogenesis, and discusses meth-

ods for assessment of liver fibrosis that are routinely used in clinical practice. Liver biopsy was until recently considered as the gold standard to diagnose and stage liver fibrosis. However, its invasiveness and drawbacks led to the development of non-invasive methods, which include serum biomarkers, transient elastography and combination algorithms. Clinical studies with CHC patients demonstrated that non-invasive methods are in most cases accurate for diagnosis and for monitoring liver disease complications. Moreover, they have a high prognostic value and are cost-effective. Non-invasive methods for assessment of liver fibrosis are gradually being incorporated into new guidelines and are becoming standard of care, which significantly reduces the need for liver biopsy.

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Key words: Hepatitis C virus; Liver fibrosis; Cirrhosis; Biopsy; Fibroscan

Core tip: Chronic hepatitis C is a leading cause of liver-related morbidity and mortality and predisposes to liver fibrosis, the excessive accumulation of extracellular matrix proteins. The staging of liver fibrosis is critical for the management and prognosis of patients. This review provides an update on hepatitis C virus (HCV) epidemiology, summarizes basic mechanisms of HCV-dependent liver fibrogenesis, and discusses common methods for assessment of liver fibrosis. While liver biopsy was until recently considered as the gold standard, novel non-invasive methods, including serum biomarkers, transient elastography and combination algorithms, are gradually being incorporated into new guidelines and are becoming standard of care.

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BURDEN OF CHRONIC HEPATITIS C: THE SCREENING DILEMMA

Chronic hepatitis C (CHC) is caused by infection with hepatitis C virus (HCV) and constitutes a major public health concern, affecting around 200 millions people worldwide^[1]. It is the leading cause of hepatocellular carcinoma (HCC) and the main indication for liver transplantation in Western countries. Although some data indicated that HCV does not increase all-cause mortality^[2], other studies postulated that CHC could reduce life expectancy by 8 to 12 years^[3,4]. Thus, HCV was reported to cause more than 86000 deaths in Europe in 2002^[5]. The mortality and morbidity attributable to CHC is expected to increase dramatically over the next 50 years, considering that the rate of new HCV infections dropped significantly only after 1989^[6]. Markov model analysis suggested that by 2030, 30% of deaths due to HCV-related complications would be preventable by increasing 50% of the patients receiving treatment with interferon/ribavirin therapy^[7]. With the development in new anti-HCV agents, including NS3/4A, NS5A and NS5B inhibitors, higher success rates for treatment are anticipated, even for patients with cirrhosis or post transplantation.

The acute infection with HCV frequently does not resolve spontaneously. Approximately 80% of the infected individuals become chronic carriers and may progress to severe liver disease. Based on the natural history of CHC it is estimated that 10%-20% of patients will develop liver cirrhosis and 1%-5% will develop HCC within 20-30 years^[8]. Once liver cirrhosis is established, HCC develops at a yearly rate of 5%-7%^[9]. Importantly, epidemiological studies have shown that most patients are unaware of their positive HCV antibody status^[6]. A report commissioned by the Institute of Medicine of the National Academies highlighted shortcomings in care for viral hepatitis, and estimated that up to 75% of patients with CHC remain undiagnosed^[10]. Along these lines, the Centers for Disease Control and Prevention (CDC) estimated that although persons born during 1945-1965 comprise approximately 27% of the United States population, they account for 75% of all HCV infections, 73% of HCV-related mortality, and are at greater risk of HCC and end-stage liver complications.

Given the fact that early diagnosis and treatment can prevent liver cirrhosis and HCC, it is reckoned that one-time testing of persons born during 1945-1965 (baby boomers) will prevent more than 120000 deaths in the United States. Based on these epidemiological data and on recent advances in treatment of CHC, the CDC is now recommending a general screening strategy with a one-time testing without prior ascertainment of HCV risk for baby boomers^[6]. A recent study showed that broader screening for HCV would likely be cost-effective^[11]. Nev-

ertheless, significant reduction of HCV-related morbidity and mortality would also require improved rates of referral, treatment and follow-up^[11]. Thus, once patients with CHC are recognized from a broader screening for HCV infection, they have to be offered appropriate clinical care and therapy. In this view, the assessment of liver fibrosis stage is the key event in clinical management of CHC, affecting both disease prognosis and treatment indication^[12].

HCV AND LIVER FIBROGENESIS: BASIC CONCEPTS

Elucidating the mechanisms underlying liver fibrogenesis is of paramount importance for management and prevention of end-stage liver disease. Liver fibrosis is defined by the excessive accumulation of extracellular matrix (ECM) proteins such as collagen, laminin, elastin, fibronectin, *etc.*, and is currently considered as a wound healing response to chronic liver injury^[13]. HCV infection directly modulates signaling and metabolic pathways by viral proteins. Moreover, it indirectly induces host antiviral immune responses leading to chronic inflammation. Together, these events promote liver fibrogenesis^[14]. The hepatic stellate cell (HSC), a vitamin A (retinoid)-storing cell residing in the perisinusoidal space of Disse, is the key fibrogenetic element. Although quiescent in the absence of inflammatory stimuli, HSCs are activated in response to liver injury and undergo transformation to proliferative, contractile myofibroblasts. Activated HSCs constitute a prevalent source of ECM production^[15] and thereby disrupt the equilibrium between deposition and dissolution of ECM proteins, which leads to fibrotic scarring and eventually to liver cirrhosis (Figure 1).

The development of cell culture and animal models that recapitulate main aspects of HCV infection and liver injury has been crucial for understanding the pathogenesis of CHC^[16,17]. This involves pathways that are implicated in the initiation and the perpetuation of HSC activation. Initiation of HSC activation is mediated by paracrine stimuli from neighboring cells, reactive oxygen species (ROS), lipopolysaccharide (LPS), or apoptotic bodies. HSCs maintain their activity in response to fibrogenetic, proliferative, chemotactic and inflammatory signaling^[14].

Direct HCV-dependent liver fibrogenesis by viral proteins

The HCV contains a positive sense single-stranded RNA that is translated to a large polyprotein precursor. The latter undergoes proteolytic cleavage by viral and host enzymes in order to generate mature structural proteins (core, E1, E2 and p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B)^[18]. These molecules may target multiple cell types, including hepatocytes, monocytes, lymphocytes and various secretory cells^[19-21], and thereby modulate cell proliferation, apoptosis, oxidative stress and innate immunity^[22].

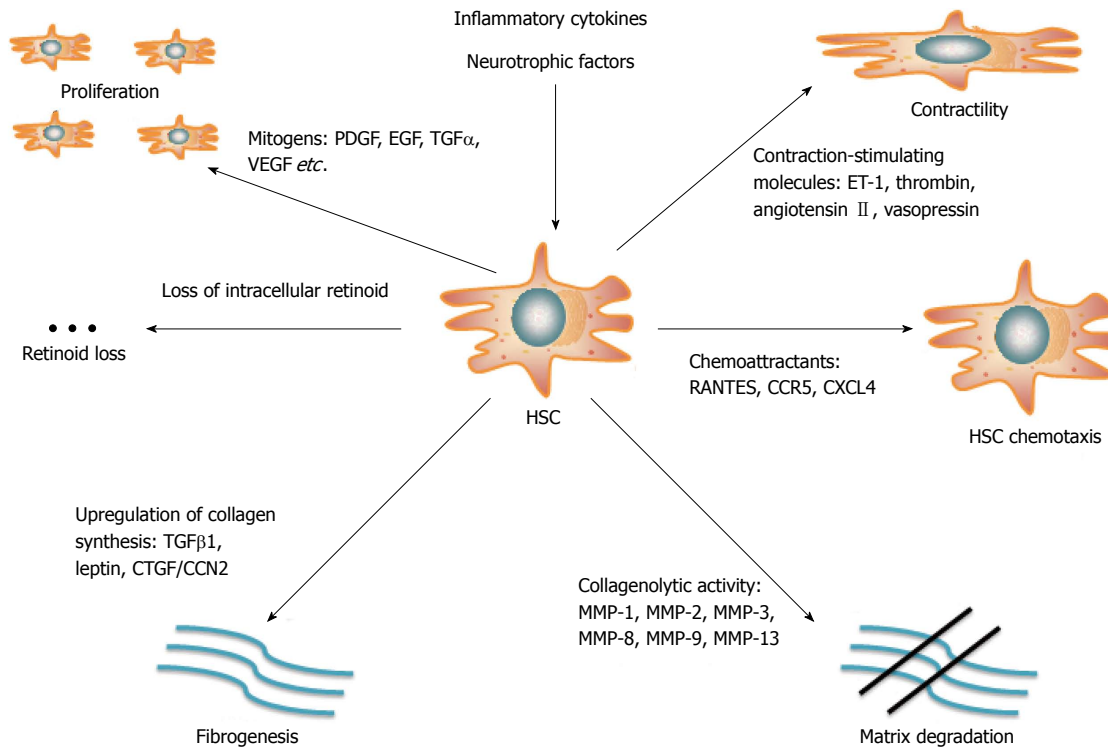


Figure 1 Hepatic stellate cells are retinoid-storing cells that play a key role in liver fibrogenesis. During liver injury, they undergo transformation from a quiescent state to proliferative, contractile myofibroblasts. Activated HSCs are the main source for production of collagen and other ECM proteins. Several molecules and pathways regulate the equilibrium between deposition and degradation of ECM proteins. HSCs: Hepatic stellate cells; ECM: Extracellular matrix; PDGF: Platelet-derived growth factor; EGF: Epidermal growth factor; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor; CCR5: C-C chemokine receptor 5; MMP-2: Matrix metalloproteinase 2.

Experimental evidence suggests that the HCV core protein, as well as non-structural HCV proteins may directly trigger HSC activation and, thus, the initiation of fibrogenesis. The core protein preferentially activates pro-mitogenic intracellular pathways within HSCs, whereas the NS3 and NS5 proteins specifically stimulate pro-inflammatory pathways *via* NF- κ B and JNK^[23]. The core and NS3 proteins promote increases in intracellular calcium [Ca^{2+}]_i and ROS levels; the effects of the core protein depend on its binding to the C1q receptor^[23]. The induction of osteopontin by calcium and ROS signaling contributes to the epithelial to mesenchymal transition of hepatocytes^[24]. The E2 glycoprotein of the HCV envelope is another potential fibrogenetic factor. It promotes the activation of matrix metalloproteinase 2 (MMP-2) upon binding to CD81 of HSCs, which results in degradation of normal ECM in areas with high HCV density, and may lead to infiltration of inflammatory cells^[25].

It should also be noted that the core, NS3 and NS5A proteins induce oxidative stress in hepatocytes and monocytes *via* activation of the NADPH oxidase^[26-28] and repression of heme oxygenase 1 (HO-1)^[29]. In addition, the core and NS3 proteins activate inflammatory pathways *via* Toll-like receptor 2 (TLR2) in monocytes, which modulate innate immunity^[30]. Furthermore, studies with HCV replicon models demonstrated the induction of oxidative stress and the activation of transforming growth factor β 1 (TGF β 1) and other pro-fibrotic signals in response to HCV replication^[31,32].

Indirect HCV-dependent liver fibrogenesis via immune responses and other pathways

The immune response to HCV infection plays a key role in the enhancement of hepatic fibrogenesis. Multiple growth factors, inflammatory cytokines and chemokines may regulate the activation of HSCs and their transformation to myofibroblasts^[33]. In particular, the immune-promoted induction of the platelet-derived growth factor (PDGF) and the subsequent mobilization of intracellular calcium elicit mitogenic effects to HSCs^[34,35]. Kupffer cell-derived transforming growth factor α (TGF α)^[36] and bile acid-induced activation of the epidermal growth factor (EGF) receptor^[37] promote the proliferation of HSCs. Moreover, induction of the vascular endothelial growth factor (VEGF) contributes to activation and proliferation of HSCs, as well as to hepatic angiogenesis, rendering this molecule a key element of the fibrogenic process^[38].

Next to the proliferative factors, fibrogenic cytokines that promote ECM production are positively regulated in the context of immune responses to HCV infection. TGF β 1 is the most potent pro-fibrotic cytokine, stimulating collagen production *via* Smad signaling^[39,40]. Moreover, additional molecules such as the connective tissue growth factor (CTGF/CCN2)^[41] and the adipokine leptin^[42] promote liver fibrogenesis *via* TGF β 1 signaling. The fibrogenic activity of leptin is partly mediated by TGF β 1 and requires further Kupffer cell-derived stimuli^[43]. Leptin also acts as a suppressor of the peroxi-

some proliferator-activated receptor γ (PPAR γ), an anti-fibrotic nuclear receptor able to abrogate HSC activation and conserve its quiescence^[44].

Chemokines enhance fibrogenesis through chemotaxis of fibrogenic cells and amplification of the inflammatory response. HSCs produce numerous receptors and secrete several cytokines^[45]; their role in the pathophysiology of fibrogenesis is currently a subject of investigation. Recent evidence suggests that the induction of C-C chemokine ligand 5 (CCL5, also known as RANTES) by the NF- κ B signaling pathway promotes chemotactic and mitogenic effects to HSCs *via* its C-C chemokine receptor 5 (CCR5)^[46]. Furthermore, platelet-derived chemokine (C-X-C motif) ligand 9 (CXCL9) exhibits anti-fibrotic properties that depend on its receptor CXCR3^[47], whereas CXCL4 exerts a pro-fibrotic function^[48].

Neurochemical and neurotrophic factors may also enhance the fibrogenetic function of the HSCs. Several cellular pathways of the neuroendocrine system are activated in response to chronic liver injury. Induction of opioid signaling by endogenous opioids stimulates proliferation of HSCs and enhances collagen deposition^[49]. Along similar lines, the activation of the CB₁ receptor by HSC-derived cannabinoids^[50], the enhancement PDGF signaling in HSCs by serotonin^[51] and the activation of HSCs by thyroid hormones^[52] promote fibrogenetic pathways.

The direct interaction of HSCs with immune cells, through expression of adhesion molecules, results in bidirectional cellular stimulation and amplification of fibrosis. Tumor necrosis factor α and monocyte chemoattractant protein 1 (MCP-1), along with other pro-inflammatory cytokines are secreted by Kupffer cells in response to NF- κ B activation^[53]. This results once again in HSCs activation and in secretion of factors that amplify the inflammatory process and perpetuate the macrophage activity, such as the macrophage colony-stimulating factor^[54], interleukin 6^[55], MCP-1^[56] and RANTES^[46]. In addition, HSCs express cell adhesion molecules including vascular cell adhesion molecule 1^[57] and intracellular adhesion molecule 1^[58]. These are involved in further recruitment of inflammatory cells in the site of injury, which enhances the fibrogenetic process. Other cell types implicated in fibrosis progression include lymphocytes^[59], macrophages^[60] and endothelial cells^[61]. Macrophages promote the survival of activated HSCs *via* NF- κ B-dependent pathways^[62]. By contrast, natural killer cells and T cells from HCV-infected patients promote apoptosis of HSCs and thereby exert anti-fibrotic function^[63].

Last but not least, oxidative stress is a key component of hepatic fibrosis^[64]. Apoptotic parenchymal cells are being phagocytosed by activated HSCs resulting in activation of the NADPH oxidase^[65]. The latter mediates the generation of ROS, which are capable of both initiating and perpetuating fibrosis *via* activation of HSCs, hepatocytes, Kupffer cells and inflammatory cells^[66]. This process is further enhanced in the presence of polyunsaturated fatty acids, ethanol and iron. Furthermore, the DNA of apoptotic hepatocytes may interact with HSCs'

TLR9 and thus enhance the collagen production and deposition^[67].

Mild to moderate hepatic iron overload is a common manifestation of CHC patients. This is largely attributed to misregulation of the iron regulatory hormone hepcidin^[68,69], which is transcriptionally inhibited by HCV-induced oxidative stress^[70]. Even though iron antagonizes HCV replication by inactivating the viral polymerase NS5B^[71,72], hepatic iron accumulation^[73], elevated serum ferritin^[74] or reduced serum hepcidin levels^[75] are associated with progression of liver disease. The hemochromatosis protein HFE, an atypical major histocompatibility complex class I molecule, may also contribute to liver fibrogenesis as an upstream regulator of hepcidin and/or as possible immunological factor^[76,77].

IMPACT OF LIVER FIBROSIS ON PROGNOSIS, MANAGEMENT AND SCREENING STRATEGIES

The accumulation of liver fibrosis is a significant incident with major consequences on the pathology development of CHC^[78]. It indicates the onset of progressive disease, which may eventually lead to cirrhosis and end-stage liver complications^[79]. Patients with absent or mild fibrosis at diagnosis have a relatively low risk (25%-30%) of developing cirrhosis over the next 20 years. Portal and septal fibrosis both cause cirrhosis, albeit with different progression rates (18-20 years for patients with portal fibrosis and 8-10 years for patients with septal fibrosis, respectively)^[80]. Thus, the stage of liver fibrosis is critical for clinical management, especially in light of the new screening wave of HCV-infected patients^[6].

The clinical management of CHC patients depends on two different stages of liver fibrosis^[81]: (1) considerable fibrosis, histologically classified as septal fibrosis (stage F3 by METAVIR), represents a definitive indication to schedule, not defer, antiviral treatment; and (2) cirrhosis (stage F4 by METAVIR) necessitates specific and regular follow-up which should include screening for HCC and esophageal varices. Apart for indication to antiviral treatment, a more advanced liver fibrosis stage should require interventions to control known negative cofactors for disease progression (Table 1). These include life style modifications (diet, weight loss, regular physical exercise), alcohol and drug abstinence, referral to specialists (hepatologist, metabolic clinics, dietician, psychologist), specific medications (statins, insulin-sensitizing agents). Thus, the new screening strategies, which are opening to a large group of persons, the baby boomers, should be associated with diagnostic and therapeutic interventions to all newly identified patients.

LIVER BIOPSY: ALL THAT GLITTERS IS NOT GOLD

For many years the assessment for liver fibrosis has been

Table 1 Factors contributing to fibrosis progression in chronic hepatitis C

Non-modifiable	Modifiable
Duration of HCV infection	High alcohol consumption (≥ 20 -50 g/d)
Older age at infection	Insulin resistance
Male sex	Obesity
Presence of baseline fibrosis	Metabolic syndrome
HIV or HBV co-infection	Daily cannabis use
Infection with HCV genotype 3	
Gene polymorphisms involved in iron overload/inflammatory pathways	
Latin ethnicity	

HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HBV: Hepatitis B virus.

through liver biopsy, which has been considered the gold standard gauge for the direct histological evaluation of the severity of liver disease.

Role of the pathologists in liver biopsy: Errors in samples and reading variability

The representativeness of liver samples obtained through a liver biopsy and the pathologist's experience remain the major determinants of diagnostic accuracy. Inadequate liver biopsy sample can lead to underestimation of liver fibrosis stage^[82]. Samples taken from both lobes of the liver in a cohort of CHC patients highlighted in 33.1% of them a difference in the fibrosis stage by at least one grade, and in 14.5% of them underdiagnosis of fibrosis^[83]. On single blind percutaneous liver biopsies, cirrhosis was missed in 10%-30% of samples^[84-86]. Since liver biopsy involves only a very small part of the whole organ (approximately 1/50000), the diagnosis of fibrosis can be missed, especially in cases where the lesions are not uniformly distributed through the parenchyma.

Misclassification of the stage of liver fibrosis can be reduced by obtaining a specimen of adequate size and quality. It has been suggested by some authors that an adequate sample of the liver should be at least 15 mm in length and ought to contain more than 5 portals^[87-89]. By critically evaluating published literature, Guido *et al*^[90] concluded that unacceptable methodological limits often flaw liver biopsy results; moreover they proposed sample sizes of 20 mm or more containing at very least 11 complete portal tracts for reliable staging. Analyzing even larger size samples going up to 25 mm in length has been suggested by other authors^[91,92]. According to the American Association for the Study of Liver Diseases (AASLD), a liver biopsy sample should contain at least 11 complete portal tracts and be no less than 20 mm in length, while liver fibrosis should be scored by a simple (METAVIR) rather than complex (Ishak) system^[93].

There is also a significant degree of inter-/intra-observer variability in the pathologic assessment of liver biopsy samples. The practical knowledge and experience of pathologists demonstrated by a longer medical career,

or affiliation within an academic realm, could have a greater influence on the interpretation of the diagnosis, more than the sample size^[94]. A pathologist with specific expertise in liver disease should interpret the biopsy, preferably in coordination with the clinician who performed the procedure and is caring for the patient. In the absence of this interaction, diagnostic errors by non-specialist pathologists have been reported in more than 25% of patients^[95,96]. If liberal use of second opinions from specialist liver pathologists has been recommended, this may result in increased costs and waiting time.

Recent studies have implied that liver biopsy should not be considered as the gold standard, but rather as the best point of reference for staging liver disease^[97,98]. Surrogates in general are evaluated by utilizing the area under the curve (AUC), with liver biopsy as the reference. Mehta and coworkers argued that the ideal surrogate will at no time attain the maximal value (1)^[97]. By taking into consideration a spectrum of accuracies of the biopsy plus a spectrum of prevalence of substantial fibrosis, they demonstrated that even under optimal conditions and with a perfect marker, it is not possible to achieve an AUC ≥ 0.90 when assessing substantial fibrosis^[97,98].

Invasiveness and cost of liver biopsy from the clinician's perspective

There are definitely advantages in performing liver biopsy since it gives important and direct information relating to fibrosis, necroinflammatory activity, steatosis stage and also hepatic iron deposits, which are recurring histological appearances of CHC and potential comorbidities. However, there are also possible drawbacks for the clinician, such as the invasiveness of the procedure and the cost (Table 2). The most frequent complication (84%) for patients undergoing liver biopsy is pain. Bleeding occurs in 0.01%-0.04% of cases, whereas death is very rarely associated with the procedure ($\leq 0.01\%$). Clinical studies have provided evidence that the rate of complications in percutaneous liver biopsy inversely correlates with the experience of the operator^[99,100], but opposite data have also been reported^[101].

There is some ongoing debate amongst physicians about liver biopsy and its role in the assessment of fibrosis. A survey with 1177 general practitioners in France showed that up to 59% of patients with CHC refused the procedure due to its invasive nature, and some 22% of the physicians had similar considerations^[102]. Liver biopsy was not performed by 29% from 112 American physicians due to following concerns: safety (72.7%), low reimbursement (66.7%), logistical issues (45.4%)^[103].

A recent Canadian nationwide survey on patterns of diagnosing liver fibrosis showed that for almost half of the physicians, liver biopsy was the main diagnostic approach. Limitations in access/availability of non-invasive tools and lack of reimbursement represented a significant barrier^[104]. A similar survey was earlier performed in France, the country where non-invasive diagnostic methods of liver fibrosis were first marketed, and ap-

Table 2 Comparison of the main characteristics of liver biopsy, serum biomarkers and transient elastography

	Liver biopsy	Serum biomarkers	Transient elastography
Advantages	Direct assessment of liver fibrosis Stage by stage fibrosis classification Evaluation of coexisting disorders (inflammation, steatosis, iron overload)	Immediate result Fast (one time blood sample) Patient friendly	Immediate result Duration of examination 5 min Operator and patient friendly
Limitations	Complications (pain, bleeding) Sampling error, intra-observer and inter-observer variability Hospitalization (day hospital) often required Cost Delayed result (2-4 wk)	Cost (unitary cost per patient for patented tests) High rates of unclassified patients (APRI, Fib-4, Forns' index, Lok index) Lower performance for diagnosis of significant fibrosis Unable to discriminate between intermediate stages of fibrosis	Cost (one time per machine) Failure in 5% of cases (25% in obese patients) Unreliable results in 15% of cases (obesity, ascites, limited operator experience) Lower performance for diagnosis of significant fibrosis Unable to discriminate between intermediate stages of fibrosis
Contraindications	Absolute: uncooperative patient, severe coagulopathy, extrahepatic biliary obstruction Relative: ascites, morbid obesity, possible vascular lesions, amyloidosis	None	Pacemaker, pregnancy
Risk factors for error	Biopsy sample < 2 cm in length, containing < 10 complete portal tracts; inexperienced pathologist	Autoimmune thrombocytopenia (APRI); Gilbert's syndrome, extrahepatic cholestasis, hemolytic anemia (Fibrotest)	Transaminases flares; acute viral hepatitis; non-fasting patient; vascular hepatic congestion; extrahepatic cholestasis; IQR $\geq 30\%$

APRI: Aspartate aminotransferase to platelet ratio index; IQR: Interquartile range.

Table 3 Role of liver biopsy and non-invasive tools across the international guidelines

Ref.	Threshold for definitive indication to antiviral therapy	Recommended methods for liver fibrosis staging	Can non-invasive methods replace liver biopsy?
APASL ^[109] , 2007	F1	Liver biopsy	No
AASLD ^[190] , 2009	F2	Liver biopsy, serum biomarkers, transient elastography	No
EASL ^[81] , 2014	F2	Liver biopsy, serum biomarkers, transient elastography	Yes
CASL ^[111] , 2012	None	Liver biopsy, serum biomarkers, transient elastography	Yes

appropriate reimbursement policies are being implemented since 2007. Interestingly, only 4% of physicians that responded, routinely requested liver biopsy^[105]. A survey among Italian hepatologists uncovered discrepancies between them on how and when to perform liver biopsy in CHC patients^[106].

Cost is a major issue for implementation of liver biopsy in clinical practice, especially in light of the recent broader screening strategies for hepatitis C. In the United States the cost is currently \$1032 and can increase up to \$2745 if complications occur during and after the procedure^[107]. In Canada, the mean cost of a complicated liver biopsy requiring hospitalization is \$4579^[108].

Liver biopsy and non-invasive tools for assessment of liver fibrosis across guidelines

Given the drawbacks of liver biopsy, non-invasive tools for assessment of liver fibrosis have attracted the attention of hepatologists. Table 3 compares guidelines in terms of recommendations for liver biopsy and/or non-invasive tools for the staging of liver fibrosis in HCV-infected patients. Overall, in spite of a previous consensus that a stage of liver fibrosis of at least F2 represents a de-

finite indication for antiviral therapy, recent guidelines recommend that there should be no threshold precluding patients from antiviral treatment. The Asian Pacific Association for the Study of the Liver (APASL), recommends treatment for patients with a histological score of F1 or above^[109]. HCV patients with viral genotypes 1-3 can be treated regardless of the stage of the disease. It is not compulsory for patients infected with HCV genotypes 2 or 3 to have a liver biopsy in order to start therapy. However, obtaining a liver biopsy before starting therapy could offer prognostic information. At the time the APASL guidelines were issued, non-invasive methods were not recommended.

AASLD guidelines state that in CHC, liver biopsy should be considered if the patient and the health care provider wish to know the fibrosis stage to enable an informed decision on treatment options and/or to predict possible outcomes. A liver biopsy may be unnecessary in persons infected with HCV genotypes 2 and 3, since more than 80% of them achieve a sustained virological response (SVR). There is, nevertheless, an ongoing argument on whether CHC patients with HCV genotype 1 warrant a biopsy because of their lower SVR rates.

Likewise, the need for liver biopsy in CHC patients with less common HCV genotypes (4-6) is unclear. At present there are accessible non-invasive tools, which might be useful in determining the absence or presence of advanced fibrosis; however they should not take the place of liver biopsy in routine clinical care practices.

More up-to-date guidelines on management of specific chronic liver diseases, give a different perspective. Thus, according to the European Association for the Study of the Liver (EASL), although liver biopsy is still the gold standard of reference in CHC, non-invasive methods may also be used instead^[110]. Similarly, the guidelines of the Canadian Association for the Study of the Liver (CASL) state that acceptable methods to stage liver fibrosis include liver biopsy, Fibroscan® and serum biomarkers^[111]. Moreover, the CASL guidelines state clearly that if F2 is a threshold for definitive candidacy to antiviral therapy, no threshold of fibrosis should preclude a patient with CHC from treatment. Overall, the diagnostic value of liver biopsy and non-invasive methods for assessment of liver fibrosis has progressively evolved across the guidelines. In the most recent ones, a clear cut-off for indication to antiviral therapy is no longer recommended. Moreover, we witnessed an evolution in the strength of recommendation of liver biopsy vs non-invasive fibrosis assessment tools, with the recent guidelines being indifferent.

NON-INVASIVE ASSESSMENT OF LIVER FIBROSIS: EPIDEMIOLOGICAL AND CLINICAL RATIONALE

The CDC guidelines recommend a onetime screening test for HCV infection in baby boomers, meaning that a new wave of identified chronic carriers will soon present in the panorama of HCV epidemiology. Once these new patients are identified, appropriate management should be offered. Liver fibrosis staging is the single most important factor impacting on the natural history of CHC. It is critical for prognosis and expedited initiation of treatment. However, it is impractical and immensely expensive to stage fibrosis through liver biopsy in all affected persons. Nowadays, this procedure should be thought of as a diagnostic funnel for large-scale screening of liver fibrosis in HCV infection. Consequently, non-invasive tools are absolutely necessary in order to restrict biopsies. In general, non-invasive methods can be divided into two main classes: the serum biomarkers, based on a biological approach; and methods based on a physical approach, including transient elastography, acoustic radiation force impulse imaging, magnetic resonance elastography. Any non-invasive method should ideally fulfill certain characteristics: it should be simple, accessible, easy interpretable, highly accurate, liver-specific, and satisfactorily validated.

The concept of validation is critical and encompasses a number of features that the ideal serum biomarker should fulfill. First, a non-invasive method should dem-

onstrate a good diagnostic accuracy. Specifically, an expensive and patented tool should demonstrate a clear advantage in terms of diagnostic accuracy when compared to simple and economic ones. Second, there should be a sufficient number of validation studies from independent researchers. Third, specific etiology-validation of the non-invasive methods should be provided considering that each etiology of chronic liver disease presents with specific pathogenesis, natural history and associated comorbidities. For example, when considering CHC and chronic hepatitis B (CHB), the former has specific associated comorbidities, such as steatosis and diabetes, the latter is characterized by a more vigorous necroinflammation^[112]. Thus, a non-invasive tool developed in the setting of CHB should be specifically validated in CHC patients. Fourth, a careful evaluation of the risk factors for error and failure of a non-invasive tool should be carried out for adequate interpretation in clinical practice. Fifth, serum biomarkers should be specifically validated in special HCV-infected populations, such as patients co-infected with human immunodeficiency virus. Finally, when dealing with serum biomarkers, particularly the patented ones, analytic conditions, such as standardization of reagents and analyzers according to manufacturer's recommendations, should be taken into account. An overview of the non-invasive diagnostic tools for liver fibrosis and their main validation features is shown in Table 4.

SERUM BIOMARKERS FOR ASSESSMENT OF LIVER FIBROSIS

There are direct and indirect serum biomarkers for assessment of liver fibrosis. The former are fragments of compounds of the liver matrix; for instance, hyaluronan, collagen synthesis or degradation products, and regulators of fibrogenetic mechanisms. The latter are biochemical parameters that can be calculated from routine peripheral blood tests. Calculations use liver-derived molecules, such as clotting factors, bilirubin, cholesterol, albumin and transaminases. Direct biomarkers mirror the metabolism of liver ECM and can be potentially utilized to assess the dynamics of liver fibrogenesis. However, they may not be routinely provided in every hospital setting, limiting their clinical use. Indirect biomarkers correlate with liver fibrosis stage. Tables 4 and 5 provide an overview of the performance of the most proven biomarkers in CHC.

Direct biomarkers of liver fibrosis

The most common direct markers investigated for liver fibrosis in CHC include laminin, hyaluronan, procollagen III, collagen type IV, YKL-40, MMPs and their inhibitors (Tables 4 and 5). Hyaluronan is a glycosaminoglycan synthesized by HSCs and degraded in the liver sinusoidal cells^[113]. In a study of 326 CHC patients, the AUC for significant fibrosis and cirrhosis were 0.86 and 0.92, respectively, and the cut off level was 110 µg/L^[113]. Nevertheless, a different cohort study involving over 400 patients

Table 4 Main validation features among the non-invasive methods for liver fibrosis diagnosis

Ref.	Parameters	Independent validation studies	Etiology-validation studies	Characterization of risk factors for error	Validation in special HCV populations
AAR ^[138]	AST, ALT	+	+	+	+
APRI ^[142]	AST, platelets	+	+	+	+
ELF ^[131]	Age, TIMP-1, hyaluronan, procollagen type III	+/-	+	+	-
Fib-4 ^[145]	Age, ALT, AST, platelets	+	+	+	+
Fibrometer ^{®[122]}	Platelets, prothrombin index, AST, α2-macroglobulin, hyaluronan, urea, age	+/-	+	+	+
Fibroscan ^{®[167]}	Liver stiffness measurement	+	+	+	+
Fibrospect ^{®[132]}	Hyaluronan, TIMP-1, α2-macroglobulin	+/-	-	-	-
Fibrotest-Fibrosure ^{®[132]}	γGT, total bilirubin, haptoglobin, α2-macroglobulin, apolipo-protein A1, age, gender	+	+	+	+
Forns' index ^[144]	Age, γGT, cholesterol, platelets	+	+	+	+
Hepascore ^[129]	Age, gender, bilirubin, γGT, hyaluronan, α2-macroglobulin	+/-	+	-	+
Hyaluronan	Hyaluronic acid	+	+	+	+
Lok index ^[191]	AST, ALT, platelets	-	-	+	-

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AAR: AST-to-ALT ratio; APRI: AST-to-platelet ratio index; AP: Age-to-platelet ratio; HCV: Hepatitis C virus; TIMP-1: Tissue inhibitors of metalloproteinases-1.

Table 5 Diagnostic performance of serum biomarkers in chronic hepatitis C

Index	≥ F2/F4						
	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR+	LR-
Hyaluronan ^[113-115,119,128]	0.73-0.86/ 0.89-0.92	64.5-75/ 79.2-100	81.0-91.2/ 80.0-89.4	44.0-86.3/ 63.0-100	78.5-93/ 99.0-100	3.94-7.32/ 5.00-7.47	0.30-0.38/ 0.00-0.23
Fibrometer ^[122,124]	0.85-0.89/ 0.91	80.5-89/ 94.1	84.1-89.9/ 87.6	82.0-86.3/ 68	77.6-82.5/ 94.7	5.56-7.97/ 7.46	0.13-0.21/ 0.06
FibroSpect ^[122,126-128]	0.82-0.87/ NA	71.8-93.0/ NA	66.0-73.9/ NA	60.9-82.6/ NA	77.7-94/ NA	2.73-2.75/ NA	0.10-0.24/ NA
Hepascore ^[124,129,130]	0.79-0.85/ 0.85-0.94	53.08-82/ 71.0-76.5	65.0-92.0/ 84.0-89.8	70-88/ 64.9	63.5-78/ 89.6-98	2.34-6.62/ 4.78-6.96	0.27-0.51/ 0.27-0.32
ELF score ^[122,131]	0.80/ NA	90/ NA	31/ NA	27.5/ NA	92/ NA	1.30/ NA	0.32/ NA
AAR ^[137,192]	NA/ 0.51-0.83	NA/ 46.7-78.0	NA/ 95.9-100	NA/ 73.7-100	NA/ 80.7-89	NA/ 19.02	NA/ 0.22-0.43
APRI ^[122,124,133,137,142,192-194]	0.69-0.88/ 0.61-0.94	41-91/ 57-89	47-95/ 75-93	61-88/ 38-57	64-86/ 93-98	1.71-8.20/ 3.56-8.14	0.19-0.62/ 0.10-0.46
Lok Index ^[137,191]	NA/ 0.78-0.81	NA/ 37-92	NA/ 30-94	NA/ 32-75	NA/ 84-91	NA/ 1.31-6.16	NA/ 0.26-0.67
Forns' Index ^[122,124,133,144,192,193]	0.60-0.86/ NA	79.8-94/ NA	61.2-95.0/ NA	66-94.7/ NA	63.8-96/ NA	2.42-15.96/ NA	0.09-0.21/ NA
Fib-4 ^[145]	0.82-0.89/ 0.79-0.91	37.6-74.3/ NA	80.1-98.2/ NA	82.1/ NA	94.7/ NA	3.73-20.77/ NA	0.32-0.63/ NA
Fibrotest ^[122,124,132,133,135]	0.74-0.87/ 0.71-0.87	65-77/ 50-87	72-91/ 70-92.9	76-80/ 57.9-93	66.7-81/ 44-90.5	2.75-7.22/ 2.9-7.04	0.31-0.38/ 0.17-0.53

AUC: Area under the curve; NA: Not available; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AAR: AST-to-ALT ratio; APRI: AST-to-platelet ratio index.

reported an AUC of only 0.73 for significant fibrosis^[114]; cirrhosis was excluded with 100% negative predictive value (NPV), a cut-off of 50 µg/L and an AUC of 0.97. In yet another study with 486 patients, hyaluronan values of < 60 µg/L were used to exclude cirrhosis with a NPV of 99%^[115]. Type IV collagen showed an AUC of 0.83 for the diagnosis of significant fibrosis^[116]. Comparison of the diagnostic performance of hyaluronan and type IV collagen revealed superiority of the former as a marker in

CHC^[117].

Laminin is a non-collagenous glycoprotein synthesized by HSCs and deposited in the liver basement membrane. The diagnostic value of laminin is not as high as those of hyaluronan and type IV collagen^[118]. Thus, a study involving 243 chronic liver disease patients reported a 77% accuracy for laminin for detecting significant liver fibrosis among a CHC subgroup^[119]. MMP-2 and tissue inhibitors of MMP-1 and -2 (TIMP-1 and -2) have also

demonstrated some diagnostic potential to detect liver fibrosis in CHC^[120].

YKL-40 is a glycoprotein that is member of the chitinase family. It is strongly expressed in human cartilage and liver, and it is involved in the fibrogenetic process. In 109 CHC patients, it showed a discrete performance for significant liver fibrosis AUC 0.81, specificity of 81% and sensitivity of 78%. However, its accuracy for the prediction of liver cirrhosis was lower, with the AUC at 0.795. A possible diagnostic value of procollagen III assessment has also been evaluated; however, it was found to be inferior compared to type IV collagen and hyaluronan^[113,121].

Direct markers have also been proposed as combination panels for increasing the diagnostic performance of the single parameter. Fibrometer[®] is a patented test combining age, platelets, hyaluronan, AST, prothrombin index, urea and α 2-macroglobulin. In CHC patients, AUC values were reported to be between 0.85-0.89 for significant liver fibrosis and 0.91 for liver cirrhosis^[122-124]. Fibrospect[®] is a combination of hyaluronan, TIMP-1 and α 2-macroglobulin that showed an AUC of 0.82-0.87 for significant fibrosis^[125-127]. A comparative study investigated the diagnostic performance of Fibrospect[®], hyaluronan and YK-40 for significant fibrosis in CHC^[128]. Interestingly, the recorded Fibrospect[®] AUC was 0.66, while that of hyaluronan was 0.76. Hepascore[®] is another patented test, combining age, gender, hyaluronan, bilirubin, γ GT, and α 2-macroglobulin. In CHC patients, AUC values of Hepascore[®] were 0.79-0.85 for diagnosis of significant fibrosis, and 0.89-0.94 for diagnosis of cirrhosis, which indicates an excellent performance^[124,129,130]. The panel of direct non-invasive markers proposed by the European liver fibrosis study group includes, hyaluronan, TIMP-1, type III collagen and age. In a cohort study involving more than one thousand patients with chronic liver disease, the panel detected significant liver fibrosis with an AUC of 0.77 in the CHC subgroup^[131].

Among the patented panels combining parameters for diagnosis of liver fibrosis, Fibrotest-Fibrosure[®] is the most validated. The parameters included in its formula are γ GT, total bilirubin, haptoglobin, α 2-macroglobulin, apolipoprotein A1, age and gender^[132]. Risk factors for error of this test include elevation of bilirubin levels unrelated to fibrosis (for example due to cholestatic or Gilbert syndromes), reduction of haptoglobin related to hemolysis, elevation of haptoglobin and α 2-macroglobulin due to non-hepatic inflammation. The number of patients that have been included in independent studies is more than 5000. The AUC values range between 0.74-0.87 for significant fibrosis and 0.71-0.87 for cirrhosis^[89,132-134]. A systematic review including 9 studies for a total number of 1679 CHC patients concluded that Fibrotest-Fibrosure[®] is excellent for its diagnostic accuracy in cirrhosis but not in early stages of fibrosis^[135].

Indirect biomarkers of liver fibrosis

Non-invasive indirect biomarkers for liver fibrosis comprise serum parameters and their combination panels,

such as platelets, transaminases, and albumin. Platelet count showed a discrete performance in ruling-out cirrhosis with a cut-off value of $150 \times 10^9/L$, with 84% to 95% NPV^[119,136,137]. The prothrombin index, based on prothrombin time, showed a NPV ranging from 82% to 91% to rule-out cirrhosis^[119,137]. However, these simple and inexpensive markers do not provide a classification of significant liver fibrosis.

One of the most adopted indirect biomarkers is the AST to ALT ratio (AAR), which is widely used for the staging of liver fibrosis in CHC patients. The normal value is < 0.8 . An increase of AAR reflects a progressive liver functional impairment, while a ratio ≥ 1 is indicative of cirrhosis^[138]. AAR distinguished cirrhotic patients from non-cirrhotic with 60%-83.6% accuracy, 31.5%-81.3% and 53%-100% specificity^[138-141]. Its performance has been variable in difference studies, and the AUC ranged between 0.51-0.83. This test is easy to perform in the daily clinical setting and it comes with no cost; however, a major limitation is that it cannot diagnose significant fibrosis, while values may be affected in case of alcohol consumption^[119].

The AST to platelet ratio index (APRI) is another simple score proposed for the classification of both significant fibrosis and cirrhosis. The APRI is calculated by using AST and platelet count, which makes it easily accessible to the clinician at virtually no cost^[142]. It is a useful tool to manifest or exclude significant liver fibrosis (cut-off 0.5-1.5) and liver cirrhosis (cut-off 1-2). However, in a substantial number of patients (30%-50%) APRI values are within an intermediate area and thus classification is unreliable. Nonetheless, to date APRI remains one of the most validated non-invasive biomarkers for liver fibrosis, and among the most referenced by guidelines^[89]. In the initial study, APRI demonstrated a high precision for the prediction of significant fibrosis (AUC 0.88) and cirrhosis (AUC 0.94)^[142]. Subsequent studies nevertheless indicated an irregular performance with AUC for significant fibrosis ranging between 0.69-0.88 and for cirrhosis between 0.61-0.94^[89,133]. This variability could be partially explained by different cut-off values chosen in each study and by population heterogeneity. A recent meta-analysis of 40 studies, which included 8739 patients with CHC, concluded that APRI can be used in clinical practice for the confirmation of severe fibrosis/cirrhosis when other clinical signs and examination are non-decisive^[143]. Moreover, since it is cheap and simple, it should be considered a reference test against which other non-invasive methods should illustrate improved precision and cost-effectiveness. Moreover, APRI is still the first choice for CHC patients to identify fibrosis in regions with limited healthcare resources.

The Lok index is a modification of APRI that combines platelet count, INR and AAR102. Cut-off values of 0.2 or 0.5 are used to rule-out or rule-in cirrhosis, respectively. Nevertheless, the Lok index is unreliable in detecting significant fibrosis. To this end, Fornis *et al*^[144] developed a simple panel based on clinical variables rou-

Table 6 Cut-off values, performance and number of patients per study of Fibroscan®

Ref.	Cut-off for \geq F2 (kPa)	Cut-off for F4 (kPa)	AUC for \geq F2	AUC for F4	Number of patients included
Sandrin <i>et al</i> ^[147] , 2003	7.6	14.4	0.88	0.99	106
Castéra <i>et al</i> ^[167] , 2005	7.1	12.5	0.83	0.95	183
Ziol <i>et al</i> ^[195] , 2005	8.7	14.5	0.79	0.97	327
Kettaneh <i>et al</i> ^[196] , 2007	6.8	17.6	0.79	0.91	935
Arena <i>et al</i> ^[197] , 2008	7.8	14.8	0.91	0.98	150
Cross <i>et al</i> ^[198] , 2010	8.9	10.1	0.89	0.97	187
Degos <i>et al</i> ^[199] , 2010	5.2	12.9	0.75	0.90	913

AUC: Area under the curve.

tinely recorded: age, γ GT, platelet count and cholesterol levels. The Forns' index utilizes cut-off values of 4.2 or 6.9 to rule-out or rule-in significant fibrosis, respectively, while intermediate values cannot be classified. A study involving 476 CHC patients revealed a high diagnostic performance of the Forns' index for the detection of significant fibrosis, with an AUC of 0.81-0.86^[144]. Remarkably, the low cut-off value of 4.2 had a NPV of 96% in excluding significant liver fibrosis. Conversely, the high cut-off value of 6.9 had a positive predictive value of only 66% in manifesting significant fibrosis. Further studies uncovered a slightly decreased performance of Forns' index, with AUC 0.76-0.79^[124,133]. The major limitation of the Forns' index is that it does not offer conclusive information regarding cirrhosis, while it leaves a high number of cases unclassified.

Fib-4 is another index combining simple biomarkers and is based on age, platelet count, AST, and ALT^[145]. Fib-4 uses cut-off values of 1.45 or 3.25 to rule-out or rule-in significant fibrosis, respectively. In a study involving 529 CHC patients, Fib-4 enabled the correct identification of cases with severe fibrosis and cirrhosis, with AUC 0.85^[145]. Similar conclusions were reached by other studies^[146]. Nonetheless, overall Fib-4 does not offer sufficient clues about cirrhosis and consistently leaves several cases unclassified. On the other hand, it is simple and cheap, and has been validated in a number of studies.

NON-INVASIVE ASSESSMENT OF LIVER FIBROSIS BY TRANSIENT ELASTOGRAPHY

The measurement of liver stiffness by transient elastography offers an accredited non-invasive method for the assessment of liver fibrosis^[147]. It is performed by using Fibroscan® (Echosens, Paris), a device composed of an ultrasound transducer probe that is mounted on the axis of a vibrator. The transducer transmits vibrations of mild amplitude and low frequency. This generates an elastic shear wave, which disseminates through the underlying tissue. Dissemination of the shear wave is monitored by pulse-echo ultrasound acquisition. Its velocity directly correlates to tissue: the faster the shear wave disseminates the stiffer the tissue. Liver stiffness is measured by Fibroscan® in a volume that is approximately a cylinder

1 cm wide and 4 cm long, between 2.5 and 6.5 cm below the skin. This volume is substantially bigger (at least 100 times) than a typical biopsy sample.

The Fibroscan® examination is painless, fast (performed in less than 5 min), and easy to use. It is performed on a patient who is lying flat on his/her back, with the right arm tucked behind the head. The probe transducer is placed on the patient's skin, in-between the rib bones at the same level as the right lobe of the liver that would be used to obtain a biopsy sample. The operator needs to acquire 10 valid measurements and then the Fibroscan® software calculates the median value. Success of each measurement is determined by the software itself. Liver stiffness ranges between 2.5-75 kPa. Fibroscan® cut-off values between 5.2-8.9 kPa are consistent with significant fibrosis, while values between 10.1-17.6 kPa indicate cirrhosis^[79,148]. Main features on Fibroscan® studies in CHC patients are summarized in Table 6.

Overall, the accuracy of transient elastography is comparable to that of patented serum biomarkers that are used for assessment of significant liver fibrosis, with AUC < 0.80. However, transient elastography shows excellent performance for the diagnosis of cirrhosis since the AUC was \geq 0.90 in all reported studies^[79]. Meta-analysis data indicate that the Fibroscan® examination alone does not provide sufficient information to diagnose significant liver fibrosis. Instead, Fibroscan® may be used together with an algorithm combining non-invasive serum biomarkers^[149]. On the other hand, the meta-analysis validated the excellent accuracy of transient elastography in the diagnosis of liver cirrhosis when other examinations and clinical signs are inconclusive. It should be noted that the French *Haute Autorité de Santé* recommends the utilization of either Fibroscan®, Fibrotest® or Fibrometer® for first line assessment of liver fibrosis in CHC patients.

Applicability of Fibroscan® in clinical practice

Even though the Fibroscan® examination *per se* is straight forward, the interpretation of the result must be done by an expert clinician, knowledgeable on the clinical background of the individual patient and on the conditions that can influence liver stiffness measurement. Factors that influence the applicability of Fibroscan® in clinical practice can be divided into three categories: (1) risk factors of failure; (2) risk factors of low quality; and (3) risk factors of false positivity.

Risk factors of failure of liver stiffness measurement include obesity, narrow intercostal space and ascites^[79]. Failure rates range between 2.4%-9.4%^[150,151]. Obesity is a major factor for failure, given its frequency in the general population. A study of 2114 examinations showed that a body mass index (BMI) ≥ 28 kg/m² was the only factor independently associated with failure^[152]. On the same line, Wong and colleagues found a failure rate of 2.6% if BMI was < 30 kg/m² and 25.5% if BMI was ≥ 30 kg/m²^[153]. To overcome the high failure rates occurring in obese patients with the Fibrosan[®] standard probe (M), a new FibroScan[®] probe (the "XL" probe) has been developed. This utilizes a hypersensitive ultrasonic transducer with a lower frequency, larger vibration amplitude, deeper focal length and higher depth of measurement. Reliable results with the XL probe were obtained in 61% of obese patients in whom the M probe failed^[154].

According to the manufacturer, the risk factors of poor quality of a Fibrosan[®] examination include an interquartile range (IQR) exceeding 30% of the median value, which reflects the variability of the validated measures, and a success rate less than 60%, that is the percentage of valid measurement. Interestingly, a study investigating 254 CHC patients showed that while IQR is indeed a factor of overestimation of liver fibrosis, success rate is not a factor significantly influencing the accuracy of Fibrosan[®]^[151].

A number of conditions can lead to false positivity of Fibrosan[®] examination. Acute viral hepatitis increases liver stiffness^[155,156]. Thus, the necroinflammatory status needs to be taken into consideration, particularly in patients with absent or low-stage liver fibrosis. In relevant studies^[155,156], ALT levels correlated with Fibrosan[®] values. Conversely, another study showed that low AST is a variable associated with discordance between Fibrosan[®] measurement and liver biopsy for diagnosis of significant fibrosis^[157]. The authors concluded that Fibrosan[®] is influenced by major variations in biochemical activity of liver disease in CHC and that liver stiffness, at low levels of AST, can underestimate fibrosis. For this reason, adjustments for age and AST of the Fibrosan[®] result may significantly improve accuracy.

In patients with extra-hepatic cholestasis, liver stiffness significantly correlates with bilirubin levels and leads to false positivity of Fibrosan[®] measurement^[158]. Fibrosan[®] value was significantly reduced following successful bilirubin drainage. Likewise, vascular hepatic congestion can erroneously increase Fibrosan[®] values. This effect is entirely reversible upon correction of cardiovascular dysfunction^[150]. Fasting is also important to avoid overestimation of Fibrosan[®] measurement. A study by Arena *et al*^[159] showed the confounding effect of a meal on the accuracy of liver stiffness in CHC patients. The authors proposed a fasting period of 120 min before performing the examination. On the same line, Berzigotti *et al*^[160] demonstrated that post-prandial hyperemia is accompanied by a marked increase in liver stiffness in patients with liver cirrhosis.

Transient elastography by using Fibrosan[®] is a highly reproducible technique^[161]. Inter- and intra-observer fluctuations are affected by high grade hepatic steatosis, mild fibrosis (F1-F2 by METAVIR) and a BMI ≥ 25 kg/m²^[161]. Nevertheless, the applicability of Fibrosan[®] may not be as good as that of biomarkers. Overall, in a study of 13369 examinations, liver stiffness data were not interpretable in nearly 20% of cases, mainly due to failure to obtain reliable measurements according to the manufacturer's recommendations. The technical limitations were attributed to obesity of patients, and in particular to increased waist circumference, and to limited experience of the operator^[162].

COMBINATION ALGORITHMS OF NON-INVASIVE METHODS FOR ASSESSMENT OF LIVER FIBROSIS

In order to increase the diagnostic performance of the single method, especially for the diagnosis of significant fibrosis, non-invasive methods have been combined in diagnostic algorithms. The rationale is to combine non-invasive methods, such as Fibrosan[®] and serum biomarkers, or different, unrelated serum biomarkers. Such a strategy led to a significant reduction in the number of liver biopsies and to an increase in diagnostic accuracy, and it has been recommended by guidelines, such as those from the EASL and CASL. In a recent review Pinzani *et al*^[163] suggested to apply two unrelated non-invasive methods in CHC patients, and to obtain liver biopsy in only one subgroup of them. On the same line, Manning and Afdhal^[164] have proposed to perform annually biomarkers analysis *plus* Fibrosan[®]. The utilization of combination algorithms does not completely eliminate the need for liver biopsies; however it can greatly reduce it and limit it to cases where serum biomarker data do not show a reliable accuracy. Combination algorithms used in clinical settings are able to provide the subsequent responses: (1) Presence or absence of significant liver fibrosis, which indicates whether to administer antiviral therapy or not; (2) Presence or absence of liver cirrhosis, which indicates whether to proceed with specific screening for esophageal varices and HCC or not; and (3) Liver biopsy needed to correctly stage hepatic fibrosis. Combination algorithms of non-invasive methods for assessment of liver fibrosis that have been proposed in the literature are summarized in Table 7.

STEPWISE COMBINATION ALGORITHMS

Sebastiani *et al*^[133,165] proposed an approach that combines APRI and Fibrotest[®] sequentially. These methods were selected because they are highly validated and widely available. The Sequential Algorithm for Fibrosis Evaluation (SAFE) biopsy was aimed at reducing the amount of liver biopsies needed to accurately stage liver fibrosis, and at minimizing misclassifications. The stepwise modeling of

Table 7 Combination algorithms of non-invasive methods for liver fibrosis proposed in chronic hepatitis C

Algorithm's name	Type	Non-invasive methods adopted	AUC for \geq F2	AUC for F4	Saved liver biopsies for > F2 (%)	Saved liver biopsies for F4 (%)	Number of studies (patients)
SAFE biopsy ^[133,165]	Stepwise	APRI, Fibrotest®	0.89-0.94	0.87-0.92	43.8-54.0	74.8-93.4	6 (4118)
Bordeaux algorithm ^[167,168]	Synchronous	Fibrotest, Fibroscan®	0.88-0.91	0.93-0.95	71.9-77.0	78.8-79.0	3 (875)
Leroy algorithm ^[124]	Synchronous	APRI, Fibrotest®	0.94	NA	19.0-29.2	NA	3 (1381)
Fibropaca algorithm ^[134]	Synchronous	APRI, Fibrotest, Forns' index	0.88	0.85	51.7	76.2-81.3	2 (1248)
Angers algorithms ^[171]	Synchronous	Fibrotest, Fibrometer®	0.892	0.917	79.8	89.7	1 (390)
Bourliere's algorithm ^[166]	Stepwise	APRI, Hepascore	91%-96% (accuracy)			33-45	1 (467)
Fibrometer® + Fibroscan ^[172]	Synchronous	Fibrometer, Fibroscan		86.7%		100	1 (1785)

APRI: Aspartate aminotransferase to platelet ratio index; NA: Not available.

the algorithms for significant liver cirrhosis and fibrosis was intended for achieving \geq 90% accuracy. The model uses APRI as a first line test because of its simplicity and low cost, and Fibrotest® as a second line test because of its accuracy and higher cost. Importantly, it uses liver biopsy as a third line test only in cases where the combined non-invasive biomarkers fail to classify with adequate accuracy. The modeling of the stepwise algorithms was established on the single biomarkers predicted values. The SAFE biopsy has been validated by data obtained in a multi-centered study with more than 2035 CHC patients (Table 7). They show excellent diagnostic performance and substantial reduction of liver biopsies (50% for significant fibrosis and 80% for cirrhosis). Another proposed stepwise algorithm combines Hepascore®, a patented test, with APRI^[166]. This approach yielded 91% diagnostic accuracy and reduced liver biopsies for significant fibrosis by 45%. To date, its main drawback is the lack of extensive validation data for Hepascore®, as compared to APRI, Fibrotest® and Forns' index.

SYNCHRONOUS COMBINATION ALGORITHMS

Castéra *et al.*^[167] proposed the Bordeaux algorithm, which combines Fibrotest® and Fibroscan®. This approach improves accuracy for the diagnosis of significant fibrosis. Performance of the Bordeaux algorithm and SAFE biopsy was subsequently compared in 302 patients with CHC^[168]. Both algorithms saved a high number of liver biopsies to diagnose cirrhosis, while the Bordeaux algorithm was more effective in the prevention of liver biopsies for the diagnosis of significant fibrosis. The accuracy of the two algorithms was similar for significant fibrosis, whereas, the Bordeaux algorithm was more accurate for the diagnosis of cirrhosis. Nevertheless, the Bordeaux algorithm requires the use of Fibrotest® and Fibroscan® in all patients, which increases cost. The SAFE biopsy is much cheaper because it requires the use of Fibrotest® only in a subgroup of patients who cannot be categorized by APRI.

Another combination algorithm consisting of Forns' index, Fibrotest® and APRI was proposed by Bourliere *et al.*^[169] and showed an exceedingly good performance for diagnosing both significant fibrosis and cirrhosis, saving

around 50% and 80% of liver biopsies, respectively. Leroy *et al.*^[124], proposed a synchronous algorithm using Fibrotest® and APRI in concordance, which demonstrated exceptional performance in the diagnosing of significant fibrosis. However, the number of saved liver biopsies was relatively small as compared to the other combination algorithms.

The SAFE biopsy, Fibropaca algorithm and Leroy algorithm were applied to 1013 CHC patients^[170]. The accuracy of the Fibropaca algorithm and the SAFE biopsy was similar; however, the SAFE biopsy reduced the number of biopsies and required the acquisition of fewer non-invasive biomarkers, thereby saving costs. Boursier *et al.*^[171] described the Angers' algorithm, which combines Fibrotest® and Fibrometer®, and showed that this could save 44.8% of liver biopsies by exhibiting an overall accuracy of 95.3%. Moreover, they suggested that the synchronous combination algorithms could be more efficient than the sequential algorithms, including SAFE biopsy, which is at present debatable. On the same line, a study of 1785 CHC patients compared the performance of eight diagnostic algorithms^[172]. The authors found an impressive 0% rate in liver biopsy need with a synchronous combination of Fibroscan® and Fibrometer®. However, even though it showed an excellent accuracy, Fibrometer® has been less evaluated independently compared to other established tests that are used for SAFE biopsy and Bordeaux algorithm (APRI, Fibroscan® and Fibrotest®), and is not licensed in as many countries as Fibrotest®.

In conclusion, combination algorithms can significantly improve the diagnostic accuracy of the single non-invasive method, particularly to diagnose significant liver fibrosis. Moreover, they can safely reduce the number of liver biopsies needed in clinical practice. The choice of the algorithm to be used in clinical practice may be based on some considerations: (1) what is locally available; (2) what is more validated; (3) what is not affected by patient co-morbidities; and (4) which methods the physicians feel more comfortable with.

MONITORING OF COMPLICATIONS IN LIVER DISEASE

Several studies suggest that complications of liver dis-

ease in compensated cirrhosis can be monitored by non-invasive techniques. As such, values of liver stiffness in cirrhotic patients increase with the progression of liver disease. In a retrospective study of 711 patients, values of liver stiffness significantly correlated with the severity of chronic liver disease in terms of Child-Pugh score, clinical parameters (ascites, varices, history of bleeding, HCC), biochemical parameters (albumin, bilirubin, platelets and INR) and other indications (large esophageal varices, splenomegaly on sonography, nodular surface, heterogeneous parenchyma)^[152]. Fibroscan® cut-off values of 27.5, 49.1, 53.7 and 62.7 kPa had > 90% NPV for large esophageal varices, history of ascites, HCC and esophageal bleeding, respectively. On the same line, Vizzutti *et al.*^[173] reported a correlation between liver stiffness and portal hypertension, as assessed by the hepatic venous pressure gradient (HVPG). A cut-off of 17.6 kPa of Fibroscan® had 90% sensitivity to rule-in esophageal varices.

In a study of 99 cases Fibrotest® showed a high NPV (100%) to exclude large esophageal varices with a cut-off value of 0.75 in detecting large varices^[174]. In another study of 70 patients, Fibrotest® showed 92% NPV for excluding large esophageal varices with a specific cut-off (0.78), with an overall AUC of 0.75; Fibroscan® showed an AUC of 0.87^[137]. A low platelet count has been related to the presence of esophageal varices. The discriminating threshold ranged between 68000 and 160000/mm³^[137,175]. However, other studies concluded that platelet count is not an adequate non-invasive marker for esophageal varices^[176]. For the diagnosis of esophageal varices, Giannini *et al.*^[177] reported an overall accuracy of 86% and good sensitivity at 91.5%, and with the cut-off platelet count to spleen diameter ratio at 909.

The value of 7 non-invasive biomarkers of liver fibrosis in prediction of esophageal varices was investigated in one study with 510 patients with cirrhosis^[175]. The presence of esophageal varices could be excluded with $\geq 96\%$ NPV by Lok index with the cut-off of 1.5. Importantly, a combination of Forns' index (8.5 cut-off) and Lok index (0.9 cut-off) could rule-out clinically significant esophageal varices, defined as varices requiring primary prophylaxis of bleeding (large esophageal varices or small varices with red signs or in Child-Pugh class C), with 91% NPV. Likewise, a good performance of Lok index for diagnosis of varices was also reported by Castéra *et al.*^[137], with a 0.87 AUC.

Complications of liver cirrhosis, including esophageal varices, ascites and hepatic encephalopathy, occur when portal hypertension develops. The gold standard of reference to diagnose portal hypertension, measurement of HVPG, is invasive and limited to highly specialized centers. Berzigotti *et al.*^[178] demonstrated that liver stiffness measurement by Fibroscan® predicts presence of portal hypertension with an AUC of 0.88 as compared to HVPG. Moreover, the performance increased significantly when Fibroscan® was combined with platelets or spleen size (up to 0.935 AUC). In a study of 100 consecu-

tive patients with CHC, spleen stiffness was demonstrated to predict accurately HVP. Moreover, a cut-off value of spleen stiffness of 41.3 was able to rule-out esophageal varices with 98% sensitivity and 66% specificity^[179].

Even though at present non-invasive methods for liver fibrosis cannot replace endoscopy for screening of esophageal varices, they may help stratifying cirrhotic patients for risk classes and possibly reducing the number of endoscopies.

PROGNOSTIC VALUE OF NON-INVASIVE METHODS FOR LIVER FIBROSIS

Evaluating the stage of liver fibrosis is a key point not only for management of the patient, but also for long-term prognosis. If CHC patients have mild fibrosis at diagnosis, only 25%-30% of them progress to become cirrhotic within 20 years. However, virtually all patients diagnosed with portal fibrosis will progress to liver cirrhosis within 18-20 years, whereas all patients diagnosed with septal fibrosis will progress to cirrhosis in only 8-10 years. Moreover, end-stage complications mainly occur in patients with advanced disease. Portal hypertension, ascites, or HCC are associated with a shorter survival. Given that the level of fibrosis predicts liver-related complications and survival, early assessment of the risk of bad prognosis helps the physician to manage patients with cirrhosis and to make decisions about liver transplantation.

Liver biopsy does not meet the criteria for serial monitoring and surrogate end-point marker tool because of its invasiveness, sampling error, intra- and inter-observer variability, cost, and patient reluctance to undergo serial monitoring. As such, the value of non-invasive methods for liver fibrosis in predicting clinical outcomes of CHC has been investigated. Ngo *et al.*^[180] showed that Fibrotest-Fibroscan® displays a significant correlation with survival, with a 5-year prognostic value similar to that of liver biopsy for the prediction of cirrhosis decompensation and survival. Along the same line, Nunes *et al.*^[181] showed that hyaluronic acid, APRI, and Fib-4 were significantly associated with mortality. An association between liver stiffness and risk of HCC development in CHC patients was also described^[182].

A definitive demonstration of the long-term predictive role of non-invasive methods for liver fibrosis comes from a study by Vergniol *et al.*^[183]. In a consecutive cohort of 1457 CHC patients, the researchers investigated the role of Fibrotest-Fibroscan®, APRI, Fib-4 and liver stiffness in predicting death, liver-related death, and liver transplantation during a 5-year follow-up period^[183]. All non-invasive fibrosis methods could predict shorter survival, with liver stiffness and Fibrotest® showing the higher predictive values. Moreover, patient outcomes worsened as liver stiffness and Fibrotest® values increased. On the same line, a recent study of 3927 patients with CHC showed that Fibrotest® and Fibroscan® predicted 10 years occurrence of severe liver-related complications, HCC,

variceal bleeding and hepatic failure^[184].

A study recently performed in our center investigated the value of Fibroscan® in diagnosing subclinical cirrhosis, as defined by liver stiffness ≥ 13 kPa and absence of thrombocytopenia, ultrasonographic signs of advanced liver disease/splenomegaly, esophageal varices, and ascites^[185]. In 1492 consecutive patients with a mean follow-up of 18 mo, we found that patients with subclinical cirrhosis had a higher incidence of cirrhosis-related events as compared to non-cirrhotic patients, including HCC. We then concluded that screening with Fibroscan® may help early identification of subclinical cirrhosis, stratifying patients by risk and establishing a surveillance program for HCC and varices.

NON-INVASIVE METHODS FOR LIVER FIBROSIS AND ANTIVIRAL TREATMENT: MONITORING, RESPONDING, REGRESSING

Antiviral therapies for CHC are medium term and expensive, and it may be clinically worthy to monitor histological data, in addition to virological and biochemical responses. Even in the rapidly changing panorama of antiviral therapy against HCV infection, the cost will remain a major issue. Initial data revealed significant alterations of Fibroscan® and Fibrotest® values in CHC patients during and after antiviral therapy. In 91 patients with CHC, Hezode *et al.*^[186] investigated the kinetics of liver stiffness during antiviral treatment with pegylated interferon alpha and ribavirin. A significant improvement in liver stiffness was observed during therapy, which continued after treatment only in patients who achieved SVR. Interestingly, similar dynamics of liver stiffness were observed in cirrhotic *vs* non-cirrhotic patients. In multivariate analysis, only the SVR was associated with long-term improvement of liver stiffness. The authors hypothesized that these changes reflect fibrosis regression. This is in keeping with reported improvement of histology in pair liver biopsies^[187,188]. On the same line, patients were more likely to achieve SVR if the baseline value of Fibroscan® or Fibrotest® was lower, and mean value of patients at end of treatment was lower in responders^[189]. Taken together, these data suggest that antiviral therapies promote regression of liver fibrosis. Larger prospective studies are required for further validation.

CONCLUSION

Staging of liver fibrosis is crucial for the management of CHC patients and for prognosis. Liver biopsy cannot be used as a screening tool due to its invasiveness and drawbacks, especially in light of recent recommendations for large scale screening against HCV infection. Non-invasive methods to stage liver fibrosis are accurate, cost-effective and patient-friendly. Combination algorithms

can help optimize the implementation of non-invasive methods in clinical practice. A rational approach is to perform a first line screening of liver fibrosis with algorithms combining the most accredited non-invasive methods and to perform a biopsy only for patients where non-invasive tests yielded unreliable or inaccurate results. Non-invasive methods for assessment of liver fibrosis can also predict cirrhosis-related complications and long-term outcomes of CHC patients. Thus, they can be used to stratify patients by risk classes and to prioritize for antiviral treatment and liver transplantation. Finally, non-invasive methods can be used to monitor the regression of liver fibrosis in response to antiviral therapy.

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