Evaluation of liver fibrosis: "Something old, something new…"

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Abstract	Hepatic fibrogenesis may gradually result to cirrhosis due to the accumulation of extracellular matrix components as a response to liver injury. Thus, therapeutic decisions in chronic liver disease, regardless of the cause, should first and foremost be guided by an accurate quantification of hepatic fibrosis. Detection and assessment of the extent of hepatic fibrosis represent a challenge in modern Hepatology. Although traditional histological staging systems remain the "best standard", they are not able to quantify liver fibrosis as a dynamic process and may not accurately substage cirrhosis. This review aims to compare the currently used non-invasive methods of measuring liver fibrosis and provide an update in current tissue-based digital techniques developed for this purpose, that may prove of value in daily clinical practice.

Keywords Non-invasive, digital, staging, histology, hepatic fibrosis

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Introduction

Hepatic fibrosis is a central pathological healing process in progressive chronic liver disease. For many years, fibrosis was thought to be irreversible. The first notion on the regression of liver fibrosis appeared in the medical literature in 1979, when Perez-Tamayo [1], analyzing the activity of liver collagenase, presented data supporting that cirrhosis could be reversible. During the last three decades, fibrosis has been widely accepted as a dynamic process with a strong potential for significant resolution. Substantial evidence originated from data showing that successful treatment of the underlying liver disorders, could reverse fibrosis and probably even cirrhosis [2-7]. Moreover, the understanding of cellular and molecular mechanisms of liver injury and insights in fibrogenesis led to the development of novel therapeutic approaches and advanced drug targets, especially for patients with chronic viral hepatitis B (CHB) or

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C (CHC). Scientific attention is currently focused on new anti-fibrotic therapies, aiming at fibrosis reversibility and cirrhosis regression [3]. It is therefore important, now more than ever, to ensure accurate and prompt assessment of hepatic fibrosis in therapeutic trials of chronic liver disease. Liver biopsy still remains the reference for assessing fibrosis, but it is now accepted that it is not a "gold standard". The dynamic process of fibrosis should be best measured as a continuous variable and classical histological staging systems do not permit this [8].

This review focuses on current histopathological and clinical challenges in the evaluation of liver fibrosis and aims to provide an update on invasive and non-invasive methods for assessing the severity of hepatic fibrosis. Furthermore, the limitations of classical tissue-based staging systems and non-invasive markers, and the advantages of emerging digital techniques that permit a more precise assessment of hepatic fibrosis are discussed.

Traditional histological staging systems

Liver biopsy incorporates information not only on fibrosis but also on inflammation, necrosis, steatosis, siderosis and other histopathological features with prognostic and predictive potential. Therefore, it still recognized as the "best standard" for the diagnosis and evaluation of fibrosis extent in chronic liver disease [8]. The first semi-quantitative histological scoring system was described in 1981 by Knodell *et al* [9], who evaluated the features of chronic hepatitis and proposed the histological activity index (HAI). HAI is an additive score calculated by summing semi-quantitative scores for four individual features: periportal and/or bridging necrosis, hepatocyte degeneration

and/or focal necrosis, portal inflammation, and fibrosis. According to HAI, fibrosis is staged using a 5-tier system, with stage 0 corresponding to absence of fibrosis and stage 4 to cirrhosis. Intermediate stages 1 and 3 correspond to fibrous expansion of portal tracts (score 1) and bridging fibrosis (score 3), respectively. To overstate the difference between mild and severe disease, Knodell et al eliminated score 2 from their system.

The histological staging systems currently in use all derive from the initial Knodell fibrosis score. These are either 5-tier (Scheuer, Batts-Ludwig, METAVIR, Brunt et al and Kleiner et al) [10-14] or 7-tier (Ishak et al) [15] and fibrosis is scored from 0-4 or 0-6, respectively (Table 1). In the vast majority of clinico-pathological studies, liver biopsies with fibrosis score ≥2/4 are considered to have "clinically significant" fibrosis [12]; cirrhosis corresponds to the highest score and the last stage in all systems.

Table 1 Traditional histological staging systems

Staging system	Stage	Histologic description	Features	Limitations
Scheuer [10]	0	No fibrosis	Preferred for CHB and CHC	Difficult distinction between stage 1 and stage 2
	1	Enlarged portal tracts	Simple in routine practice	Unclear description of stage 3
	2	Periportal fibrosis±periportal septa		Inclusion of both periportal fibrosis and portal-portal septation in stage 2
	3	Architectural distortion, but no obvious cirrhosis		
	4	Cirrhosis (probable or definite)		
Batts-Ludwig [11]	0	No fibrosis	Simple, reproducible, validated in clinical practice	No evaluation of regression/remodeling
	1	Portal/periportal fibrosis	Preferred for CHB and CHC	No evaluation "beyond cirrhosis"
	2	Septal fibrosis		
	3	Bridging fibrosis with architectural distortion		
	4	Cirrhosis		
METAVIR [12]	0	No fibrosis	Simple, reproducible, validated in clinical practice	All systems appoint "numerical" scores to each stage
	1	Portal fibrosis without septa	Extensively used	Inappropriate use of numerical calculations for a continuous variable
	2	Few septa		No evaluation of regression/remodeling
	3	Numerous septa without cirrhosis		No evaluation "beyond cirrhosis"
	4	Cirrhosis		
Ishak et al [15]	0	No fibrosis	Preferred for research purposes	Increased inter- and intra-observer variability
	1	Expansion of some portal areas with or without septa	Still reproducible and validated in clinical practice	
	2	Expansion of most portal areas with or without septa	7-tier scale has more discriminant descriptive power	
	3	Expansion of most portal areas with portal-portal bridging		
	4	Expansion of most portal areas with portal-portal and portal-central bridging		
	5	Bridging with occasional nodules		
	6	Cirrhosis		
Laennec [16]	0	No fibrosis	Sub-staging of cirrhosis	Limited validation
	1	Minimal fibrosis	Histologic substages of cirrhosis are related to clinical cirrhosis stages	Does not address disease etiology
	2	Mild fibrosis		Overlapping features within stage 3-4 related to septal thickness
	3	Moderate fibrosis		
	4A	Cirrhosis, mild or probable		
	4B	Cirrhosis, moderate		
	4C	Cirrhosis, severe		
Brunt et al [13]	0	No fibrosis	Developed for NASH	Cannot be applied in simple NAFLD
	1	Zone 3 (perisinusoidal, focal or extensive)	Evaluation of central-based fibrosis	Cannot be applied in pediatric NAFLD
	2	Zone 3 as above and focal/extensive portal-based fibrosis		No evaluation "beyond cirrhosis"
	3	Same as 1 or 2 with bridging fibrosis		
	4	Cirrhosis		
Kleiner et al [14]	0	As per Brunt et al [13] but stage 1 is further subdivided in	Covers the whole spectrum of NAFLD, including simple NAFLD	No evaluation "beyond cirrhosis"
	1	1a: delicate zone 3 sinusoidal/pericellular fibrosis (z3s/pf)	Can be applied to pediatric and some cases of obesity-related NAFLD	5-tier scale has less discriminative power
	2	1b: dense z3s/pf		
	3	1c: portal fibrosis only		
	4			

CHB, chronic hepatitis B; CHC, chronic hepatitis C; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis

Sub-staging of cirrhosis

In 2002, Ian Wanless, then at the University of Toronto, Canada was the first to attempt sub-classification of cirrhosis [16]. His proposal was based on the evidence that cirrhosis may substantially regress or may even be reversible in a variety of liver disorders. The Laennec scoring system, a modification of the METAVIR system, subdivides stage 4 (cirrhosis) into three sub-stages (4A, 4B and 4C), taking into consideration the width of the fibrous septa and the size of cirrhotic nodules. This histological sub-classification is clinically important, since hepatologists now recognize that all types of cirrhosis are not the same. A clinical sub-classification of liver cirrhosis based on disease pathophysiology, hepatic venous pressure gradient (HVPG), and the compensation status of the cirrhotic patient was proposed in 2010 [17]. Indeed, histological sub-staging of the "last stage" correlates well with the clinical sub-stages of cirrhosis, the grade of portal hypertension [18,19], and patient prognosis [20].

Non-invasive assessment of liver fibrosis

In the past decade, several non-invasive methods for assessing hepatic fibrosis have been published, resulting in more non-invasive tests than histologic scoring systems. The non-invasive tests were introduced to estimate the likelihood of advanced liver fibrosis in patients with chronic viral liver disease at presentation, and on follow up to assess fibrosis regression post-treatment [21]. These tests were later applied in alcoholic (ALD) [22,23] and non-alcoholic fatty liver disease (NAFLD) [24-26]. There are three general categories of non-invasive tests for liver fibrosis: 1) serologic panels or tests; 2) combinations with other serum tests and/or clinical features (such as age and gender) in complex algorithms; and 3) imaging-based techniques [27].

Today, non-invasive methods are widely available. Their most important advantages are the absence of contra-indications and dangerous complications for the patients, and their reproducibility [28]. In contrast to liver biopsy, many non-invasive methods can effectively evaluate fibrosis extent in the whole organ and not only in a part of it. Their potential ability to identify and differentiate between advanced fibrosis stages, the high specificity and sensitivity to diagnose cirrhosis, and their easy application makes them a useful tool in daily clinical practice. Their role becomes more significant because their diagnostic accuracy can be increased if they are combined; i.e. a serological panel may be used in conjunction with an imaging technique [29,30].

Serologic panels

The serologic fibrosis markers are broadly categorized into direct and indirect [28]. Direct markers of fibrosis include indices reflecting collagen synthesis or collagen degradation. The best-validated marker is hyaluronic acid (HA), a glycosaminoglycan synthesized by hepatic stellate cells (HSCs) [31]. HA levels correlate with fibrosis in ALD [32] and chronic viral hepatitis [33-35] and a highly negative score may be used in clinical practice as a reliable index for exclusion of fibrosis. Amino-terminal propeptide of type III collagen is a marker associated with collagen deposition and its levels are increased in acute and chronic hepatic diseases [27]. Tissue inhibitors of metalloproteinases (TIMPs/TIMP-1, TIMP-2), on the other hand, associated with the procedure of collagen degradation, which is a progressive to fibrosis consequence [27].

Indirect markers of fibrosis are simple routine blood tests reflecting alterations in liver function but not directly representing extracellular matrix metabolism. These biomarkers include indices related to portal hypertension (platelet count, spleen size), liver synthetic parameters (i.e. albumin), liver enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), AST/ALT ratio, γ-glutamyltransferase (γ-GT), bilirubin and others (Table 2). They can be used in combination to produce serologic panels such as PGA (prothrombin time; γ-GT; and apolipoprotein) and APRI (AST to Platelet Ratio Index), described below. PGA is one of the first biological indices used for the noninvasive detection of cirrhosis in alcoholic liver disease patients [36]. APRI is based on serum AST level and platelet count. It is calculated as (AST/upper limit of normal*) x100/platelet count and has been extensively studied in patients with HCV or ALD [28,37] (*adjusted according to the reference values of each laboratory).

Combined scores and algorithms

The long list of scores combining direct and indirect serum fibrosis markers and clinical data that have been developed

Table 2 Non-invasive methods for assessment of liver fibrosis

Direct serum markers	Indirect serum markers/panels	Combined scores/algorithms
Hyaluronic acid	AST/ALT	Fibrotest®
Laminin	γ-GT	Hepascore®
YKL-40	Platelet count	Fibrospect
Procollagen type III	Albumin	Fibrometer®
(PIIINP)	PGA	Forns score
Metalloproteinases MMP-1, MMP-2	APRI	ELF
TIMPs		SAFE biopsy
TGF-β1		FIB-4 score
MP3 index		NAFLD fibrosis score

YLK-40, chondrex, human cartilage glycoprotein-39; PIIINP, procollagen III amino terminal peptide; TIMPs, tissue inhibitors of metalloproteinases; TGF-β1, transforming growth factor-β; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GT, gamma-glutamyl transferase; PGA, (P, prothrombin time; G, gamma-glutamyl transpeptidase; A, apolipoprotein AI); APRI, AST to platelet ratio index; ELF, European liver fibrosis panel; SAFE, sequential algorithm for fibrosis evaluation; FIB-4 score, fibrosis-4; NAFLD, nonalcoholic fatty liver disease

and validated in recent years indicate the continuing need for increased accuracy in non-invasive evaluation of fibrosis. The currently used clinical combined scores, referred to also as "algorithms" in the literature, are summarized below.

Fibrotest® (Fibrosure® in the USA): It is the most studied and extensively validated algorithm. It involves five serum parameters (apolipoprotein-A, a2-macroglubin, γ-GT, total bilirubin, and haptoglobin) and takes into account patient age and gender. Fibrotest has been primarily used for patients with chronic viral hepatitis and is commercially available [38].

Hepascore: Four serum parameters (bilirubin, γ-GT, HA, and a-2-macroglobulin), patient age and gender are assessed in Hepascore, an algorithm mainly studied in chronic viral hepatitis. Hepascore can predict fibrosis with a trustworthy AUROC 0.81 for significant fibrosis and 0.88 for cirrhosis [39].

Fibrospect: Fibrospect uses a combination of serum HA, TIMP-1, and a-2-macroglobulin, in order to discriminate patients with moderate or severe fibrosis, from those without fibrosis, especially in CHC [40].

Fibrometer: Fibrometer includes six individual blood indices (platelet count, prothrombin index, AST, a-2-macroglobulin, HA, and blood urea) and combines them to predict severe fibrosis in chronic viral hepatitis [41].

Forns score: Forns score derives from the combination of platelet count, γ-GT, cholesterol, and patient age. It has been validated in CHC as well as in chronic hepatitis of non-viral etiology [42].

European Liver Fibrosis panel (ELF): Score based on the combination of HA, TIMP-1, and amino-terminal propeptide of type III collagen. ELF has been studied in many different patient cohorts and has been proved to be a useful tool in various chronic liver diseases, especially in ALD and NAFLD [43].

The combination of the APRI index with Fibrotest, a sequential algorithm referred to as "SAFE Biopsy" [44,45] (SAFE: Sequential Algorithm for Fibrosis Evaluation), can improve the diagnostic accuracy of the aforementioned panels, by detecting significant fibrosis (≥ F2 by METAVIR) and cirrhosis (F4) in CHC patients. The SAFE biopsy identified significant fibrosis and/or cirrhosis with >90% accuracy [44], moreover, it dismissed the need for liver biopsy in approximately 50% of the patients when it was used to identify significant fibrosis, and in >80% when it was used to detect cirrhosis. The stepwise combination of APRI and Fibrotest with liver biopsy, in CHB patients, indicated that non-invasive tests and liver biopsy, when used together as agonists, may improve diagnostic accuracy when assessing in the assessment of hepatic fibrosis [45].

BAAT score [body mass index (BMI), age, ALT, triglyceride (TG) levels]: This is the first index developed for the assessment of NAFLD fibrosis. It is calculated by summing four specific features, assigning 1 point for each of the following: BMI ≥28 kg/m², age ≥50 years, ALT ≥twice the normal values, and TG ≥1.7 mmol/L. According to Ratziu *et al*, a score of 0 or 1 excludes significant fibrosis with negative predictive value (NPV) of 100% [46].

BARD score: BARD was validated in a cohort of NAFLD. It combines 3 variables: AST/ALT ratio, BMI, and the presence of type 2 diabetes. According to Harrison *et al*, a score 0 or 1

has a very high (96%) NPV for advanced fibrosis. However, as expected, variables such as obesity, diabetes and age, influence the score, resulting in a very low positive predictive value (PPV) [47].

NAFLD fibrosis score: NAFLD fibrosis score is calculated combining six variables using the following logistic formula: -1.675 + 0.037 age (years) + 0.094 x BMI (kg/m²) + 1.13 x impaired fasting glucose/diabetes (yes=1, no=0) + 0.99 x AST/ALT ratio - 0.013 x platelet (x10⁹/L) - 0.66 albumin (g/dL). Values under the cut-off of -1.455 may exclude advanced fibrosis, whereas values higher than 0.676 are indicative of advanced fibrosis [48,49].

FIB-4 score: FIB-4 score is one of the best-validated non-invasive tests for the assessment of fibrosis in patients with advanced NAFLD [47,48]. According to a study of 541 patients with NAFLD fibrosis, FIB-4 score had a very high NPV (90%) in excluding and a satisfying PPV (80%) in diagnosing fibrosis. The calculating formula is: (Age x AST)/(Platelet count(x10⁹) x√ALT). In daily clinical practice, NAFLD score and FIB-4 are used to determine the necessity of liver biopsy in NAFLD patients [50].

Imaging techniques

In recent years, a wide spectrum of imaging techniques, based on classical tools such as ultrasonography (U/S), computed tomography and magnetic resonance imaging have improved the specificity for the detection and assessment of hepatic fibrosis. These include the following:

Transient elastography (TE) (Fibroscan®-Paris, France): TE is the most widely used imaging method for non-invasive and rapid measurement of hepatic tissue stiffness [51]. TE uses a probe that consists of an ultrasonic transducer and a vibrator that emits low-frequency shear waves (50 MHz) propagating into the liver tissue. The speed of the shear waves is directly related to liver tissue stiffness and units are expressed in kiloPascal (kPa). Many studies have evaluated the diagnostic accuracy of TE for diagnosing cirrhosis with specificity and sensitivity approaching 90%. The accuracy for fibrosis detection is lower, with sensitivity and specificity approaching 70-80% [52-54]. Obesity, ascites, acute inflammation, liver congestion, and elevated portal vein pressure may reduce TE accuracy, because both adipose tissue and the presence of fluid may influence the velocity of the shear wave [27,28,55]. Furthermore, a falsely increased liver stiffness, due to postprandial increase in portal vein pressure, has been observed [56,57].

Magnetic resonance elastography (MRE): MRE evaluates liver stiffness by measuring the propagation of mechanical waves [58]. These are produced by an active probe, placed on the patient's back, directly over the liver. As a result, the magnetic scanner generates an elastogram, acting as a guide to quantify liver stiffness. MRE is superior to TE because of its ability to scan the whole organ and its application in patients with ascites or obesity. The main drawbacks are the high cost and complexity of the method that is too procrastinating for daily clinical practice. MRE values may be affected by the increased portal vein pressure following a meal, similar to TE [59].

Acoustic radiation force impulses (ARFI): ARFI uses conventional hepatic U/S to assess liver stiffness [60]. ARFI uses short duration acoustic pulses that produce mechanical excitation. The speed of the produced waves correlates directly with the extent of liver fibrosis and results are expressed in m/sec. Advantages of this technology include the ability to select the area to be assessed, avoiding large vessels or ribs [28] and the fact that steatosis does not influence the accuracy of the procedure.

Real-time sonography-based elastography: This method estimates the velocity of a shear wave through the liver using U/S and the results are expressed in kPa [61].

2D-Shear wave elastography (2D-SWE): 2D-SWE combines U/S images with radiation force induced into the liver. 2D-SWE can measure shear waves propagation in real time [62]. Advantages of 2D-SWE (expressed either in m/sec or in kPa) include good applicability and adjustable region of interest depending on the operator.

Contrast-enhanced sonography: This technique is based on intravenous injection of specifically sized microbubbles, transferred with a shell of protein or biopolymers that facilitate their sonographic imaging [63]. The time needed for the microbubbles to pass through the liver (hepatic vein transit time) is proportional to the underlying liver fibrosis and is lower in patients with cirrhosis.

Clinical research aims on gradually increasing the diagnostic ability of non-invasive methods for assessing fibrosis; Castera *et al* [29] proposed an algorithm for decision making based on the combination of TE and Fibrotest® to accurately evaluate fibrosis and cirrhosis. Using the algorithm for the diagnosis of significant liver fibrosis in patients with CHC [30], a large number of biopsies (approximately 75%) can be avoided. The combination of a serum-based test with an imaging technique is another step in the continuous trial to improve the accuracy of non-invasive techniques. Although the use of these algorithms requires evolutional computerized systems they still remain a promising tool in clinical practice.

Digital tissue-based methods for assessing liver fibrosis

In the last decade, the increasing need for better diagnostic accuracy of tissue-based methods for evaluating fibrosis has led to the development of digital tools. The most popular quantitative method for measuring the extent of fibrosis in Sirius red-stained liver tissue sections using computer-assisted digital image analysis [64] is based on the evaluation of collagen proportionate area (CPA) [65]. The equipment includes a digital camera connected to a personal computer and specific software using a gray-scale slider that selects the overall tissue area and calculates this in pixels. Subsequently, with the aid of a red-green-blue threshold, the areas of Sirius red-stained collagen are also expressed in pixels. The "fibrosis ratio" between the two areas is expressed as the relative proportion (%) of collagen in the liver tissue or CPA. To eliminate image artifacts, fibrous tissue close to the liver capsule and large blood vessels is excluded from the measurements.

CPA has been validated as an accurate tool for quantifying hepatic fibrosis in cirrhotic and non-cirrhotic patients. Importantly, CPA gains a place in the endeavor for accurate histological assessment of fibrosis as a continuous variable, in contrast to current histological staging systems, which assess fibrosis semi-quantitatively and assign non-continuous stages. Recent data show that CPA may assess patient prognosis as it predicts liver-related outcomes including clinical decompensating events [66,67]. In patients with recurrent hepatitis C after liver transplantation, CPA was shown to be more accurate in predicting fibrosis regression and clinical decompensation compared to Ishak staging [64]. In the same study, CPA significantly correlated with HVPG values. Independently of biopsy length, CPA showed a significant correlation with HVPG cut-off values that are diagnostically important; the ability of CPA to discriminate liver fibrosis progression and therefore to distinguish "early" from "late" cirrhosis was even greater in the lower HPVG values (early portal hypertension). Thus, CPA and HVPG measurements could complement each other for a more accurate reflection of cirrhosis severity, supporting CPA as a superior tool to subclassify cirrhosis.

Steps in the future of tissue-based fibrosis evaluation

Imaging data of supramolecular structures obtained by a multiphoton microscope is an innovative and much promising technique in modern pathology. It may be used to precisely quantify and score fibrillar collagen structures, without staining, using endogenous sources of nonlinear signals [68]. Two-photon excitation fluorescence (TPEF) and second harmonic generation (SHG) can be very helpful in this direction. Fibrillar collagen has the important biological property of a high crystalline triple-helix structure, which bereaves centrosymmetric organization at microscopic and mesoscopic scales. Second harmonic microscopy seems to be a major step ahead in the accurate evaluation of liver fibrosis by precisely quantifying non-stained fibrillar collagen and enabling the evaluation of fibrosis progression.

A group of pathologists in France [69] scored fibrillar collagen deposits, using the fibrosis-SHG index that describes the correlation between the evaluation of collagen deposits and the imaging data from the SHG signal. They demonstrated a perfect correlation between the METAVIR fibrosis score and the fibrosis-SHG index in different fibrosis stages (F0-F4). The study cohort included patients with CHB and/or CHC. The method allowed the discrimination not only between patients with advanced fibrosis versus cirrhosis, but also between advanced fibrosis versus no fibrosis (F0-F1). Necroinflammation does not affect SHG scoring.

Most recently, Xu *et al* devised a method based on the technology of SHG/TPEF [70]. They developed the "qFibrosis index" based on specific parameters of histopathological architectural features and the changes of collagen patterns. The method was applied in CHB patients. They employed a list of 87 collagen architectural features, categorized into three groups:

septal collagen, portal collagen and fibrillar collagen. The samples were imaged in a SHG/TPEF technology-based microscope and the combination of pathology-relevant collagen structures with the automated computer-assisted image analysis produced "qFibrosis index"; a trustworthy quantitative index, which could reliably recapitulate METAVIR staging scores. qFibrosis values increased proportionately with fibrosis progression and the differences between all stages were significant, indicating that "qFibrosis index" may be a useful tool in subcategorizing cirrhosis. Classical histological staging systems, such as Knodell's or Ishak's, can be translated into qFibrosis, with the presupposition that they incorporate similar architectural features [65]. The accuracy of qFibrosis may be increased by co-implementing TPEF or other imaging techniques, in order to provide information not only on fibrosis but also on other histopathological features, such as necroinflammation and steatosis [70]. A recent study by Pirhonen *et al* tested SHG microscopy in the assessment of liver fibrosis in NAFLD patients. In this first description of SHG imaging in NAFLD, use of the automated SHG microscopy system improved the sensitivity of fibrosis detection in NAFLD, especially in early stages [71].

Overview and critical analysis

Liver biopsy is an invasive and frequently painful procedure that may rarely be led to dangerous complications, such as intra-peritoneal bleeding and hemobilia, with a reported mortality of 0.009 to 0.12% [8,72,73]. Liver biopsy assesses a small tissue core corresponding to only about 1:50,000 of the whole organ, so there is a risk of under- or over-estimation of fibrosis in the entire organ (sampling error) [74]. Other limitations include inter-observer variability and higher cost compared to most non-invasive techniques for fibrosis assessment [75,76] (Table 3).

The traditional histological staging systems are semi-quantitative methods, assigning numerical algorithms without quantitative relation to the underlying liver disease [77]. Despite their recognized value in routine histopathological practice they are inadequate to sub-classify cirrhosis [66]. Although all systems are well validated for everyday use they have potential disadvantages. In the Scheuer system [10], for example, differences between "enlarged portal tracts" (stage 1) and "periportal fibrosis" (stage 2) may be subtle and the pathologist may not recognize these with ease, while the meaning of "architectural distortion, but no obvious cirrhosis" is ambiguous. Furthermore, the inclusion of "periportal fibrosis" and "portal-portal septa formation" in the same category is a major drawback because only the latter is recognized as "clinically significant fibrosis" (≥F2 by METAVIR). All systems appoint "numerical" scores to each stage. However, the use of numerical calculations for a continuous variable, as is fibrosis, is now thought conceptually inexact, as already noted in the Introduction [77].

On the other hand, there are many crucial issues regarding the use of non-invasive tools (Table 3). Serum markers of fibrosis are not liver-specific and they may be affected by the presence of other factors, such as inflammation; they actually represent the rate of matrix turnover and not matrix deposition. Therefore, it is inevitable that high inflammatory activity will result in increasing their values. Likewise, absence of inflammation may lead to underestimation of fibrosis [30,78-80]. In addition, serum markers are well validated only in chronic viral hepatitis (mostly in CHC and less in CHB) and less studied in ALD and NAFLD; in chronic liver disease of other etiology they are still not validated [62]. Moreover, the fact that serum markers are surrogates and not biomarkers reduces their accuracy [27].

Novel imaging technology despite its increasing accuracy still has limitations. In addition to high cost and limited local availability for some of the methods, indeterminate results regarding the presence or absence of advanced fibrosis are reported to occur in 14-33% of cases [27]. Furthermore,

Table 3 Advantages and limitations of liver biopsy and non-invasive methods for the evaluation of fibrosis in chronic liver disease

	Advantages	Limitations
Liver biopsy	Reference tool for assessing fibrosis	Histological staging assesses fibrosis as a non-continuous variable
	Detailed information on liver histopathology	Invasive procedure with rare complications
	Subclassification of cirrhosis (Laennec staging/CPA)	Sampling variability/evaluation of a tiny part of the whole organ (1/50,000)
		Inter-/intra-observer variability
		High cost
Non-invasive methods	No contraindications	Direct markers are not liver-specific
	No complications	May be affected by other factors (i.e. TE: inflammation, congestion)
	Increased accuracy for excluding cirrhosis/advanced fibrosis	Direct markers assess matrix turnover not deposition
	Some are currently available in routine settings	Not widely validated in routine clinical practice and in a wide range of chronic liver diseases (applied mainly in CHC)
		"Grey zone" (indeterminate results in 14-33% of cases)
		High cost and limited local availability for some imaging techniques

CHC, chronic hepatitis C; CPA, collagen proportionate area; TE, transient elastography

intermediate stages of fibrosis cannot be predicted [62]. Most non-invasive tools for liver fibrosis assessment have yet to be validated in routine clinical practice [30,78-80].

The use of non-invasive methods may reduce the number of liver biopsies performed, but still cannot completely replace the need for obtaining liver tissue for histological evaluation of fibrosis [8,81-83]. In cases with cirrhosis or absent/minimal fibrosis, the stepwise combination of non-invasive tools can provide accurate results avoiding liver biopsy. However, a liver biopsy will be necessary to accurately stage fibrosis in indeterminate cases with non-invasive scores in the "grey-zone" [28,84].

The development of digital (CPA) and new innovative (SHG/TPEF, qFibrosis) tissue-based methods as objective and accurate staging tools has increased their value in recent years. Moreover, CPA has significant clinical applications, especially in assessing the response to anti-fibrotic therapies in cirrhotic patients. Initial clinical data show that cirrhosis can be accurately subclassified using CPA. In addition, combination of CPA with other continuous variables, such as HVPG, may provide useful information for predicting decompensation in patients with recurrent hepatitis [66,67,84,86].

Based on the above, CPA may be a better index of fibrosis progression and clinical outcome-predictor compared to TE [66,67]. In future studies, combination of CPA with TE may create a unique histological fibrosis index with increased accuracy. Limitations of CPA include its application to a rather small range of chronic liver diseases to date [87] and the need of larger sample size for reliable results (22-28 mm²), depending on the etiology of underlying disease [88]. Although CPA appears to be of high value for subclassifying cirrhosis, the non-standardized image analysis methods, limited validation and high cost of the equipment imped its applicability in routine practice [66,67,85].

SHG microscopy has all the advantages of CPA but with fewer limitations. It is fast and easy to perform since it does not require specific stains and its measurements are not affected by sample size [69]. SHG uses 3D imaging data of fibrillar collagen fibers overcoming the limitations of 2D imaging of a classical liver biopsy. In addition, SHG imaging can assess extracellular matrix remodeling and may prove useful for evaluating the response to anti-fibrotic treatment and fibrosis regression.

Similarly, qFibrosis may play a significant role in the near future to assess for the presence of cirrhosis and estimate fibrosis regression in CHB patients treated with new long-term antiviral regimens [70]. The possible combination of qFibrosis with non-invasive imaging tools, such as TE, could enhance their accuracy.

Concluding remarks

The scientific and clinical progress in our understanding of liver fibrosis provide hope for successful anti-fibrotic therapies in the near future. Accurate evaluation of liver fibrosis is of paramount importance in assessing post-treatment regression; to achieve this ultimate goal, well-validated methods of fibrosis

evaluation are required. Serum biomarkers, clinical algorithms and imaging techniques have become widely available and applied in clinical practice and their significance for diagnostic and follow-up purposes in the era of direct acting antivirals is increasing. Digital tissue-based methods are invaluable in accurately assessing fibrosis progression/regression and architectural remodeling influencing treatment decisions in chronic liver disease.

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