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Applying Non-Invasive Fibrosis Measurements in NAFLD/NASH: Progress to Date

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Abstract

Nonalcoholic fatty liver disease (NAFLD) has now become a worldwide health issue due to the obesity epidemic, affecting approximately 90% of the obese population and 15–40% of the general population. It is the most common form of chronic liver disease in the United States. NAFLD constitutes a spectrum of diseases ranging in severity from mild, such as fatty liver, progressing into nonalcoholic steatohepatitis (NASH), then fibrosis, and ending with cirrhosis. NASH and increasing fibrosis stage are associated with increased morbidity and mortality; the fibrosis stage is therefore a critical element of risk stratification needed to determine therapeutic approach and also the response to treatment. Liver biopsy is considered the 'gold standard' in the diagnosis of NAFLD. However, it is not practical for widespread clinical use because it is invasive, costly, and associated with complications including occasional death. These limitations have driven the development of noninvasive tests that can accurately predict the fibrosis stage in those with NAFLD. In this review, we provide a concise overview of different non-invasive measurements used for NAFLD/NASH.

Key Points

Noninvasive tests to evaluate fibrosis rely on either changes in the physical properties of the liver or on changes in levels of specific chemical moieties reflective of fibrogenesis.

Simple non-invasive laboratory aids such as FIB-4 and APRI can be used to rule out clinically significant fibrosis in those with chronic liver disease.

Tests with higher specificity are being developed to increase the positive predictive value of testing and enhance the ability to rule in advanced fibrosis. Vibration-controlled transient elastography and magnetic resonance elastography are often used for this purpose.

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1 Introduction

Nonalcoholic fatty liver disease (NAFLD) has emerged as a leading cause of liver-related morbidity and mortality. It is estimated that the contribution of NAFLD to the burden of end-stage liver disease will triple by 2030 both in the United States and globally [1]. Most of this will be due to nonalcoholic steatohepatitis (NASH), a more aggressive clinical-histological phenotype of the disease [2]. There is, therefore, an urgent unmet need to develop strategies to prevent and treat this condition.

A successful strategy for the control of NAFLD as a public health threat has to include methods for risk assessment and stratification and development of approaches for prevention and treatment that are appropriately escalated with increasing risk. Furthermore, once intervention is initiated, effective methods to assess the success of the treatment in routine clinical settings must also be available. To date, histological assessment of the liver using a liver biopsy has served as the reference standard for evaluation of the presence and severity of NAFLD as well as for the assessment of disease progression and regression [3]. While histological assessment has provided major insights into disease evolution, its utility for routine clinical assessment of NAFLD is severely limited by its invasive nature, associated morbidity and occasional mortality, and substantial intra- and inter-observer variability in assessment. These

limitations underscore the need to develop simple noninvasive methods for the assessment of disease status in those with NAFLD.

There are two principal components of histological assessment that inform the clinical approach to management of NAFLD. The first is the assessment of disease activity, which refers to the biological processes that are injuring the liver and driving its progression towards cirrhosis. This generally refers to the metabolic substrate overload in the liver and resultant cell stress, cell death, and inflammatory response that drives fibrogenic remodeling towards cirrhosis. Under the microscope, this is defined by the presence of steatohepatitis rather than steatosis and by histological activity scores such as the NAFLD activity score (NAS) and the steatosis-activity-fibrosis (SAF) scores [4]. The second key histological readout is the fibrosis stage, which defines how far the disease has actually progressed towards cirrhosis [5]. Since fibrosis stage is proximate to cirrhosis, it is not surprising that fibrosis stage reflects mortality risk more clearly than markers of disease activity [6]. It is therefore a central aspect of risk assessment and determination of who should receive pharmacological therapy and is also a key read out of the efficacy of therapeutics for NASH [1, 6]. Fibrosis typically starts in perisinusoidal regions (stage 1) and then progresses to include portal areas (stage 2), followed by formation of central vein–central vein or central vein–portal tract fibrotic bridges (stage 3), culminating in cirrhosis (stage 4) (Fig. 1). In this review, we will provide an update on current efforts on the development of noninvasive methods for the assessment of fibrosis in those with NAFLD.

2 General Aspects of Biomarker Development and Fibrosis Assessment

A biomarker is an objective patient characteristic that is measured as an indicator of a normal biological process, pathogenic process, or the biological response to a therapeutic intervention [7]. While multiple methods can be used to evaluate a biomarker, the methods themselves do not constitute the biomarker. Biomarkers must also be fit for purpose and the data needed to validate their use must be considered in this context.

A key element in biomarker development is the context of use (COU). The COU defines the boundaries within which the biomarker is to be used, the specific question it will address, its interpretation, and the decisions that will emanate from the biomarker result. These are ultimately used to qualify a biomarker for a specific purpose by the US Food and Drug Administration (FDA), which is required for its widespread availability and adoption in clinical practice. It also defines the clinical conditions and populations in which the biomarker's utility is assessed. In addition, before engaging in extensive biomarker qualification efforts, it is imperative to define the robustness of the analytic methods to be used and the conditions under which the biomarker can be tested. Finally, the biomarker must provide benefit to the patient in terms of improved sensitivity or specificity of diagnosis or prognosis; conversely, the risk of misclassification by the biomarker must be taken into consideration. For example, the potential for harm to the patient is much greater if a biomarker fails to identify the patient with cirrhosis vis-à-vis a biomarker that fails to diagnosis NASH. Thus, where the risks due to failure to diagnose a condition are high, high degrees of specificity and sensitivity are both needed, whereas in screening studies where the potential for harm from misclassification is low, a high sensitivity alone may suffice. Finally, the specific approach for analysis

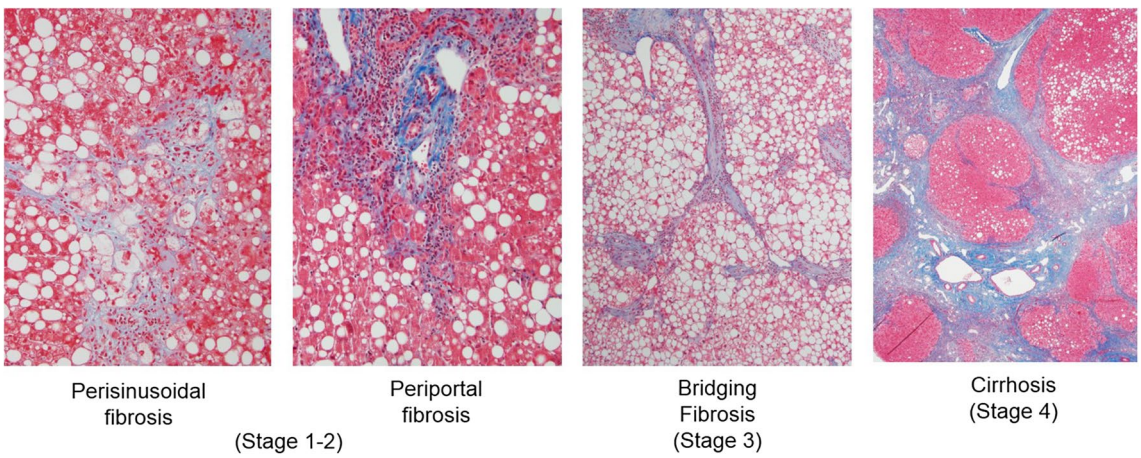

Fig. 1 Representative images of liver histology showing stages of fibrosis. Courtesy of David E. Kleiner, M.D., Ph.D. National Cancer Institute

of data for biomarkers when the reference standard is flawed remains an area where additional innovation is needed; the best practices for such analyses have been extensively reviewed and are beyond the scope of this paper and interested readers are referred to recent papers and FDA guidance documents (https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM628118.pdf?) on biomarker qualification for NAFLD/NASH [8].

Unfortunately, much of the published literature is suboptimal with respect to the quality and quantity of evidence needed to qualify biomarkers for fibrosis due to NASH. There are two large consortia in Europe and in North America that are now engaged in rigorous trials to generate the evidence needed (https://litmus-project.eu/). In the following sections, we provide what is currently known about existing biomarkers for fibrosis (Table 1).

3 Circulating Biomarkers

Biomarkers may broadly be considered as those measured in circulation versus those that rely on the physical/chemical properties of the liver. Circulating biomarkers may further be considered as those identified by unbiased evaluation of circulating biomaterials such as plasma proteins, lipids, and small metabolites, cell free nuclear material (RNA and DNA), microRNA (miRNA), microparticles and exosomes or biomarkers using a specific set of analytes. Biomarkers may also be considered to be either early in development or in advanced stages of development.

3.1 Biomarkers Based on 'omics'-Based Technologies

3.1.1 Lipidomics

There are several studies that have evaluated the possibility of identifying the stages of fibrosis based on the circulating lipidome [9, 10]. The technical robustness of these assays and the feasibility of scale up for commercial use remains to be fully defined. With development of NAFLD, the ratio of circulating n9/n7 fatty acids change reflecting increased stearoyl CoA desaturase activity, which is dependent on activation of SREBP-1, a key insulin-sensitive transcriptional factor. There is also an increase in cholesterol synthesis, reflected in an increased desmosterol:total cholesterol ratio [11]. Furthermore, there is activation of multiple lipoxygenases that are expected to increase the inflammatory state [12, 13]. These include 5-hydroxyeicosatetraenoic acid (5-HETE), 8-HETE, and 12–15 HETE as well as oxidized lipids such as 12-hydroxyoctadecadienoic acid (12-HODE) [14]. There is an accompanying depletion of arachidonic acid from multiple lipid classes as well. Decreased phosphocholine has also been reported in some individuals [15]. However, these results are largely exploratory and a rigorously validated lipidomic signature reflective of the fibrosis stage does not currently exist. This remains an area of active investigation.

Bile acids are a special class of lipids and are derived from hepatic metabolism of cholesterol and further modified in the intestine. Bile acids not only help with lipid digestion but are important nutrient sensors and regulators of metabolic homeostasis. An increase in primary bile acids and decrease in secondary bile acids is an early feature of NAFLD, and worsens with progression to NASH and increasing fibrosis, with greatest depletion of secondary bile acids in cirrhosis [16]. While these changes are seen in total primary and secondary bile acids, additional specific changes with a shift towards trihydroxylic bile acids, which are less potent than dihydroxylic bile acids for FXR and TGR5, also occur [17]. Also, despite a decrease in total secondary bile acids, deoxycholate levels increase and have been linked to hepatocellular carcinoma (HCC) development [18]. The accurate measurement of bile acids is, however, challenging and the assays are not yet robust enough for large-scale deployment. Further, the specificity and sensitivity of these changes for diagnostic purposes remain to be well established.

3.1.2 Proteomics

Proteomics technologies have also evolved, allowing measurement of a large number of proteins in a small plasma sample. The ability to develop a limited set of proteins and establish a platform for their measurement for high throughput clinical laboratories remains an open question at this time. However, a large, very well done study using a proteomics platform from Somalogic, Inc. in a population-based study in Iceland demonstrated several proteins that were linked to specific gene variants and further shown to be related to clinical outcomes [19]. This provides proof of concept that a proteomic-based approach can be used to identify subpopulations with specific clinical phenotypes and outcomes. We have recently found several differentially expressed proteins in a small cohort of subjects with NAFLD covering the full spectrum of disease [20]. These identified several proteins linked to fibrogenesis, immune regulation, and other proteins of unclear function that were related to increasing fibrosis stage (Table 2). Validation studies in independent cohorts are currently under way.

There have also been many attempts to identify a circulating signature of NASH based on circulating cytokines. A clear association between insulin resistance associated adipokines such as leptin, tumor necrosis factor-alpha (TNF-α), adiponectin, resistin, and visfatin and NAFLD as well as NASH has been shown [21]. However, the specificity of these findings and their ability to differentiate between

Table 1 Summary of non-invasive scoring systems based on biochemical markers in NAFLD/NASH

Noninvasive test/score	Components	Disease	COU	AUC	Cut-off values for advanced fibrosis
NAFLD fibrosis score (NFS) [33]	Age, hyperglycemia, BMI, platelet count, albumin, AST/ALT ratio	NAFLD fibrosis	Diagnostic	0.82 0.68	NFS < − 1.455 = F0–F2 NFS 1.455–0.675 = indeterminate NFS > 0.675 = F3–F4
FibroTest [52]	Bilirubin, GGT, a2-macroglobulin, haptoglobin, apolipoprotein A1	NAFLD fibrosis	Diagnostic	0.86	Fibrotest > 0.30 = advanced fibrosis (≥ F3)
APRI [43]	AST, platelets	Fibrosis, cirrhosis in mixed patient population	Diagnostic	0.82	APRI > 1 = advanced fibrosis (≥ F3)
FIB-4 [34]	Platelets, ALT, AST and age	HCV fibrosis	Diagnostic	0.76	FIB-4 < 1.30 = F0–F1 FIB-4 > 2.67 = advanced fibrosis (≥ F3)
Enhanced liver fibrosis (ELF) test [55]	Hyaluronic acid, tissue inhibitor of matrix metalloproteinase-1, amino terminal propeptide of procollagen type III	NAFLD in children Chronic liver disease	Diagnostic	0.92–0.99 0.80	ELF > 0.3576 = advanced fibrosis (≥ F3)
BARD score [44]	BMI, AST/ALT ratio, type 2 diabetes mellitus	NAFLD fibrosis	Diagnostic	0.67	BARD score > 2 = advanced fibrosis (≥ F3)
Fibrometer [53]	Platelets, prothrombin index, aspartate aminotransferase, α2-macroglobulin (A2M), hyaluronate, urea, and age	Viral and alcoholic chronic liver diseases fibrosis NAFLD	Diagnostic	0.883 0.943	FibroMeter for NAFLD > 0.49 = significant fibrosis (≥ F2)
HAIR [49]	Hypertension, ALT, IR index	NAFLD	Diagnostic	0.90	HAIR score ≥ 2, has specificity 89% and 80% sensitivity for the screening of NAFLD at the stage of steatohepatitis
NASHTest [127]	α2-macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin, GGT, fasting glucose, triglycerides, cholesterol, ALT, AST, age, sex, weight, height	NAFLD	Diagnostic	0.79	NASHTest scores of 3 and 4: borderline (probable) NASH Scores of ≥ 5 = NASH
ADAPT [128] (PRO-C3-based fibrosis algorithm)	Age, diabetes, PRO-C3, platelet count	Advanced NAFLD fibrosis	Diagnostic	0.87	ADAPT score ≥ 6.3287, has 91% sensitivity and 73% specificity for the identification of significant fibrosis F2–4
Hepascore [131]	Bilirubin, GGT, hyaluronic acid, α2-macroglobulin, age, and sex	HCV	Diagnostic	0.85	Score ≥ 0.5 is 92% specific and 67% sensitive for significant fibrosis, a score < 0.5 is 81% specific and 95% sensitive for advanced fibrosis, and a score < 0.84 is 84% specific and 71% sensitive for cirrhosis

ALT alanine aminotransferase, *APRI* AST to platelet ratio index, *AST* aspartate aminotransferase, *AUC* area under the curve, *BMI* body mass index, *COU* context of use, *GGT* γ-glutamyl transferase, *HCV* hepatitis C virus, *IR* insulin resistance, *NAFLD* non-alcoholic fatty liver disease, *NASH* non-alcoholic steatohepatitis, *PRO-C3*N-terminal pro-collagen III peptide

NAFL and NASH or to accurately define disease activity or stage are not sufficiently high enough for them to be used as 'stand-alone' biomarkers or biomarker panels. Recently, decreased insulin-like growth factor-binding protein 3 (IGFBP3) and increased interleukin (IL)8 has been associated with NASH, lobular inflammation, and ballooning in adults and children [22, 23]. High plasminogen activator inhibitor-1 (PAI-1) has been associated with definite NASH whereas IL8 is associated with steatosis and fibrosis in children with NAFLD [24]. In this study, soluble IL2 receptor was directly associated with portal inflammation and fibrosis stage while IL7 was inversely associated with these histological features [24]. Recent studies have further focused on markers of inflammasome activation to be associated with progressive NASH [25]. The limitations of cytokine-based biomarkers as standalone tests noted above are also relevant for these more recently identified cytokine associations with NASH, inflammation, and fibrosis. The logistical issues of robustness and reproducibility of assay performance, diurnal variability, etc. all remain to be well established for cytokine measurement methods.

3.2 Microbiome and Microbiome-Derived Metabolites

Very early data from a single-center study has identified several microbiome-derived metabolites that are associated with advanced fibrosis [26]. The same study also identified specific changes in the intestinal microbiome, assessed in stool samples, that were linked to advanced fibrosis (bridging fibrosis or cirrhosis). Specifically, increased representation from the phylum Proteobacteria and decreased representation from the phylum Firmicutes has been linked to advanced fibrosis [27]. It is, however, worth noting that such changes are seen in obesity and the sample size of the study was not sufficient to distinguish between the collinearity between the risk factors for NASH and advanced fibrosis and the advanced fibrosis itself. This also remains an area of very active investigation.

3.3 Cell-Free Nuclear Material, miRNA and Microparticles

With improvements in technology, the possibility of a 'liquid biopsy' allowing measurement of cell-free DNA, RNA, miRNA, and separation of exosomes and microparticles is now a reality. The robustness of these assays and the feasibility of scaling up the methods for high throughput use remains unclear at this time. However, early studies indicate the potential for these to be related to both disease activity and stage in NASH [28–30]. Of these various biomarkers, there is considerable interest in identifying miRNA that may be reflective of fibrosis stage. We have previously demonstrated multiple miRNA that are differentially expressed in NASH [31]. Of these, miR122 is increased in the circulation as a function of worsening liver injury and is associated with depletion of miR122 in the liver [32]. miR34a also increases in circulation but in contrast to miR122, its expression in the liver increases with increasing injury. Both of these are linked to lipid metabolism and there is biological plausibility to support these changes. Much further work is needed to establish an miRNA-based diagnostic signature of fibrosis in NASH.

3.4 Biomarkers Based on Specific Candidate Panels

Multiple biomarker panels have also been developed to determine fibrosis stage. Several of them are commercially available and have been validated for diseases other than NAFLD. Their utility for the assessment of fibrosis in NAFLD is discussed below.

3.4.1 Routinely Available Laboratory Aids

3.4.1.1 NAFLD Fibrosis Score In 2007, Angulo et al. introduced the NAFLD fibrosis score (NFS) [33]. The score is calculated from clinical and lab variables, which include age, hyperglycemia, body mass index, platelet count, albumin, and aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio. The score was efficient in detecting advanced fibrosis with high accuracy (82% in the validation group) by applying the high cutoff score (0.676). On the other hand, the score could be used for exclusion of advanced fibrosis (88% in the validation group) by applying the low cutoff score (− 1.455) [33]. This score was not found to be sensitive to change in the FLINT (farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis) clinical trial [126].

3.4.1.2 FIB-4 This test was developed as a marker of fibrosis in those with HIV-hepatitis C virus (HCV) co-infection [34]. Vallet-Pichard et al. reported FIB-4 as an accurate marker of fibrosis in HCV infection validated against a liver biopsy [35]. The score is calculated from routinely available variables including age, ALT, AST, and platelets. In the study by Shah et al., FIB-4 was validated for use in NAFLD when its performance was compared against other non-invasive fibrosis markers in NAFLD patients [area under the receiver operating characteristics (AUROC) with advanced fibrosis (F3–F4) was 0.802] (95% CI 0.758–0847) [36]. Positive predictive value was 80% for FIB-4 score of 2.67, negative predictive value was 90% for score of 1.30. Similar

Table 2 Proteomic studies of non-alcoholic fatty liver disease

Studies	Protein	Mechanism or function	Implications
Cai et al. [42] 2005 Wang et al. [91] 2015 Lim et al. [92] 2014	NF-κB TNF-α IL-1 MAPK1 JNK	Inflammation	Intracellular oxidative stress, cholesterol and triglyceride metabolism, and insulin resistance
Lorena et al. [93] 2004 Rashid et al. [94] 2012 Fitzpatrick and Dhawan [95] 2014 Younossi et al. [96] 2005	Fibrillin TGF-β CYR61 Wnt-5a Lumican Fibrinogen-γ FGF2	Fibrosis	Pathogenesis of fibrosis Marker of a profibrotic state in patients with NAFLD
Choe et al. [88] 2013	Apolipoproteins	Protein carrier	ApoB/ApoA1 ratio can be a marker of cardiovascular risk in NAFLD
Gray et al. [89] 2009	CD5L		Distinguish between different stages of NAFLD in addition to factoring in NAFLD progression
Rodríguez-Suárez et al. [97] 2010	CPS1	Metabolic pathways	Decreased in NASH compared with control patients
	GRP78		Increased lipogenesis promotes NAFLD
Sirota et al. [98] 2013	Uric acid		Increased serum uric acid levels may be associated with severe NAFLD Mitochondrial oxidative damage may play a role in NAFLD pathogenesis
Haukeland et al. [99] 2006	CCL2/MCP1	Immune cells and cytokines	Increased levels in NASH Potential role in recruitment of immune cells
Bell et al. [100] 2010 Alkhouri et al. [101] 2009 Graham et al. [102] 2006 Christou et al. [103] 2012	RBP4		Promotes fibrogenesis in NAFLD Possible association with insulin resistance, T2DM, and metabolic syndrome
Yoneda et al. [104] 2007	hs-CRP	Acute phase Reactant	Predictive markers of hepatocyte injury and NAFLD progression Increased levels in NASH
Yu et al. [105] 2012	Hemoglobin		
Kamada et al. [106] 2013	Fuc-Hpt		
Yoneda et al. [107] 2008	PTX-3		
Kaneda et al. [108] 2006	Hyaluronic acid	Extracellular matrix	Promotes fibrogenesis (not specific to NAFLD)
Yoneda et al. [109] 2007	Type IV collagen 7S		Progression of NAFLD can be partly related to unregulated fibrogenesis and remodeling
Gabrielli et al. [110] 1996	Laminin		
Krishnan et al. [111] 2012	Lumican		Different pathophysiological pathways of protease and adipocyte involvement in NAFLD progression
D'Amico et al. [112] 2010 Wanninger et al. [113] 2011	MMP-9		
Kwak et al. [114] 2012	Bilirubin	Anti-inflammatory and antioxidant	Inverse relationship between total serum bilirubin level and the prevalence of NAFLD Bilirubin can potentially act as an antioxidant anti-inflammatory substance
Mansouri et al. [115] 2010 Kessova et al. [116] 2003	SOD2 SOD1		Plays role in hepatic ATP formation and protection of mitochondrial DNA
Jayaraman et al. [117] 2005 Yamaguchi et al. [118] 2004 Zhu et al. [119] 2014 Sabapathy et al. [120] 2004	Cytochrome B5 Annexin A5 Annexin A6 Bax Caspase 3 Caspase 8	Apoptosis	Important role in the pathogenesis of NAFLD

Table 2 (continued)

Studies	Protein	Mechanism or function	Implications
Anderson and Borlak [121] 2008 Al Sharif et al. [122] 2014 Ehx et al. [123] 2014	SREBP-1 ChREBP-1 PPARγ Acetyl-CoA Glycerol-3-phosphate Fetuin A Fetuin B	Lipid synthesis	Oxidative stress plays an important role in NAFLD pathogenesis Promote lipid-induced insulin resistance
Binas and Erol [124] 2007 Higuchi et al. [90] 2011	FABP-1	Lipid metabolism	Facilitative role in hepatic fatty acid oxidation

ATP adenosine triphosphate, *CCL2/MCP1* CC-chemokine ligand 2/monocyte chemo-attractant protein-1, *ChREBP-1* carbohydrate-responsive element-binding protein 1, *CPS1* carbamoyl phosphate synthase 1, *CYR61* cysteine-rich angiogenic inducer 61, *FABP-1* fatty acid binding protein 1, *FGF2* fibroblast growth factor 2, *hs-CRP* high sensitivity C-reactive protein, *IL-1* interleukin 1, *JNK* c-Jun N-terminal kinase, *MAPK1* mitogen-activated protein kinase 1, *MMP* matrix metalloproteinases, *NAFLD* non-alcoholic fatty liver disease, *NASH* non-alcoholic steatohepatitis, *NF-κB* nuclear factor κB, *PPARγ* peroxisome proliferator-activated receptor gamma, *PTX-3* pentraxin 3, *RBP4* retinol binding protein 4, *SOD1* superoxide dismutase 1, *SOD2* superoxide dismutase 2, *SREBP-1* sterol regulatory element-binding protein 1, *T2DM* type II diabetes mellitus, *TGF-β* transforming growth factor beta, *TNF-α* tumor necrosis factor α, *Wnt-5a* wingless-type MMTV integration site family, member 5A

studies supported these findings when comparing with other non-invasive markers of advanced fibrosis in NASH [37–40]. Interestingly, FIB-4 can also be predictive of mild to moderate fibrosis in NAFLD. A cutoff of 1.43 had AUROC 0.821 (95% CI 0.75–0.891) to detect stage 1 fibrosis or higher [41]. The FIB-4 test is also sensitive to change [36]. The FIB-4 test is a robust and moderately accurate clinical aid that can identify those with advanced fibrosis as well as exclude those with clinically significant fibrosis (stage 2 or higher) with accuracy [130].

3.4.1.3 AST to Platelet Ratio Index (APRI) The APRI is a simple method that was initially developed by Wai et al. to predict fibrosis in HCV-infected patients [43]. In a retrospective cohort study of patients with NAFLD, an APRI score > 1 had sensitivity and specificity of 30% and 92.8%, respectively, with likelihood ratio of 4.2 for significant fibrosis [38]. On the other hand, the APRI score was less favorable in detecting advanced fibrosis (AUROCs 0.67–0.78, 37, 46, 47) based on other studies. When compared with FIB-4, APRI score is not a good discriminator between intermediate stages of fibrosis in NAFLD patients with a more inferior predictive value in NAFLD compared with HCV (AUROC of 0.8307 vs 0.9965) [129]. Therefore, this index is best studied in fibrosis related to HCV. As such, there is less enthusiasm for the use of APRI in the context of NAFLD.

3.4.1.4 BARD Score Harrison et al. developed the BARD score from a retrospective cohort of patients with NAFLD [44]. The score is calculated from the sum of body mass index (BMI), AST/ALT ratio, and diabetes mellitus (DM). For advanced fibrosis (≥ F3), a score of 2–4 had an AUROC of 0.81 in the validation group. The score had an odds ratio of 17 (95% CI 9.2–31.9) for advanced fibrosis, with a negative predictive value of 96% if the score is 0–1. The BARD score reportedly had a high negative predictive value ranging from 81 to 97% [45–47]. On the other hand, Ruffillo et al. reported a sensitivity of 51.4% and specificity of 77.2% for advanced fibrosis [46]. This finding may be attributed to BMI and DM [48].

3.4.1.5 HAIR HAIR score constitutes a combination of clinical and laboratory data based on hypertension, ALT, and insulin resistance. The AUROC is relatively high (0.90), sensitivity is 80%, and specificity is 89%. However, the use of this score is limited to severely obese patients; therefore, the biggest drawback is that it could not be validated externally for other NASH patients [49].

3.4.1.6 NASH Resolution Score One study found that a simple scoring system (called NASH Resolution Score) can accurately predict steatohepatitis resolution after 52 weeks of lifestyle changes [125]. The score consists of weight loss, type 2 DM, a NAFLD activity score ≥ 5, normal levels of ALT at the end of intervention, and age. By applying two predefined cutoffs, the vast majority of patients can be correctly classified as achieving steatohepatitis resolution or not. This study reported that the degree of weight loss was identified as the most consistent and robust predictor of NASH resolution.

3.4.2 Commercial Laboratory Tests

3.4.2.1 FibroTest/FibroSure® The FibroTest (FT) (also commercially known as FibroSure) is a score that can be used in a variety of liver diseases. The test comprises the following markers: total bilirubin, γ-glutamyl transferase (GGT), a2-macroglobulin, haptoglobin, and apolipoprotein A1 corrected for age and sex [50]. The test was previously vali-

dated as a marker for advanced fibrosis in hepatitis B and C. ActiTest is another sub-test that uses ALT for measuring the necro-inflammatory activity in chronic viral hepatitis patients [51]. Ratziu et al. reported that FT had an AUROC of 0.81 for advanced fibrosis (stage 3–4) and an AUROC of 0.88 for cirrhosis (stage 4) in NAFLD. A lower FT cut-off of 0.30 had a 90% negative predictive value for advanced fibrosis (sensitivity of 77%) while a higher FT cut-off of 0.70 had a 73% positive predictive value for advanced fibrosis (specificity of 98%) [52]. The limitation of this test is that it may not be easily applicable because it requires several variables that are not routinely measured in most laboratories.

3.4.2.2 Fibrometer Fibrometer is a test that consists of serum markers including platelets, prothrombin index, aspartate aminotransferase, α2-macroglobulin, hyaluronate, and urea, and clinical parameters that include age and body weight [53]. It can evaluate fibrosis based on underlying etiology. There are fibrometers for viral hepatitis, alcoholic liver disease, and NAFLD [54]. This marker was reported to have a high diagnostic performance. A study that compared the Fibrometer for NAFLD with NFS and APRI revealed that the Fibrometer had higher AUROC for significant fibrosis (≥F2) of 0.943 (95% CI 0.91–0.98) compared with the NFS 0.885 (95% CI 0.83–0.93) versus the APRI 0.866 (95% CI 0.81–0.92) [53].

The Fibrometer for NAFLD also demonstrated excellent accuracy when three intervals of fibrosis were applied: F0/F1 (95% accuracy), F0/F1/F2 (75% accuracy), F2/F3/F4 (87.9% accuracy) with an overall accuracy of 91.9%. Overall, the diagnostic performance of Fibrometer for NAFLD was better for intermediate fibrosis stages (F1/2) and less robust for advanced fibrosis (F3/4).

3.4.2.3 The European Liver Fibrosis Test (ELF)™/Enhanced Liver Fibrosis (ELF) Test The ELF test consists of a combination of serum markers for hepatic metabolism, including hyaluronic acid (HA), tissue inhibitor of metalloproteinase-1 (TIMP-1), and propeptide of type III procollagen (PIIINP) [55]. The ELF test is a modified version of the Original European Liver Fibrosis Test [7]. It has been validated in a variety of chronic liver diseases, including NAFLD [56]. It is a highly sensitive test that can be utilized as a screening test for advanced fibrosis and cirrhosis [56]. Using the ELF score may decrease the need for biopsy by ~ 60% [53]. The ELF test was successful in identifying advanced fibrosis (≥F3) in a validation cohort of 196 patients, with an AUROC of 0.90 (95% CI 0.84–0.96). A threshold of 0.3576 had a sensitivity of 80%, specificity of 90%, positive predictive value of 71%, and negative predictive value of 94% for severe fibrosis [57]. In the UK, the ELF test has been recommended by the National Institute of Clinical Excellence in their NAFLD management guidelines due to its accuracy in distinguishing advanced fibrosis and cost effectiveness compared with other serum biomarkers or imaging studies such as vibration controlled transient elastography (VCTE) or magnetic resonance elastography (MRE) [58]. It has, however, not yet met FDA standards for qualification as a diagnostic or prognostic biomarker and remains to be approved for these purposes. Similar to FT, use of the ELF test can be limited because it is based on markers not routinely available.

3.4.2.4 Nordic Bioscience PRO-C3 and PRO-C6 The extent of extracellular matrix (ECM) turnover can be determined through measurement of circulating collagen fragments with epitopes released during collagen formation and degradation. Examples of collagen fragments secondary to collagen formation include PRO-C3 (N-terminal pro-collagen III peptide), PRO-C5 (C-terminal pro-peptide of type V collagen), PRO-C6 (C-terminal pro-peptide of type VI collagen), and basement membrane biomarker P4NP7S (7 S domain of type IV collagen). Collagen fragments reflecting collagen degradation include C3M (matrix metalloprotease [MMP] degradation fragment of collagen III) and C4M (MMP degradation fragment of collagen IV) [59–61].

We have recently reported the results of a cross-sectional study of PRO-C3–6 as static markers of fibrosis stage [62]. These indicate that PRO-C3 is a moderately accurate biomarker of fibrosis stage. However, the overall accuracy was not substantially superior to conventional laboratory aids such as FIB-4. This is not totally surprising because PRO-C3 is a collagen fragment that is reflective of neoepitopes exposed in collagen fragments released during collagen biogenesis [63]. Thus, it may be a better marker for fibrogenic drive rather than the fibrosis stage itself [64]. Elevated baseline PRO-C3 levels (≥ 20.2 ng/mL) is predictive of progression of fibrosis in chronic hepatitis C [64, 65]. Further, PRO-C3, PRO-C5, and C4M have also been correlated with hepatic venous pressure gradient for prognosis of portal hypertension [66, 67].

4 Biomarker Based on Physico-Chemical Properties of the Liver

4.1 Elastography-Based Assessment of the Liver

Several methods have sought to take advantage of the stiffening of the liver with increasing fibrosis to obtain a noninvasive assessment of hepatic fibrosis stage. These include magnetic resonance elastography or soundwave-based methods such as transient elastography, shear wave elastography and acoustic radial force impulse (ARFI) [68–71]. The underlying physics for these methods are

variable and interested readers are referred to recent reviews of the subject for details [71–74].

Transient elastography is the most studied method in the context of NASH. It has the advantage of being deployed in the clinic, making it convenient for use. A plethora of data demonstrate its ability to predict underlying fibrosis stage in viral hepatitis. Its use in NASH is limited by the difficulty in interrogating the liver when the skin-to-liver distance increases due to obesity. The XL probe overcomes this problem by providing better coverage with skin-to-liver distances of up to 5 cm [75]. While early studies reported unreliable results in many subjects, successful measurement of liver stiffness in over 95% of patients with NAFLD with a high level of inter-observer concordance was reported by the NASH Clinical Research Network recently [76]. This method can identify those with advanced fibrosis (bridging or cirrhosis) and those with minimal fibrosis with accuracy but is less accurate in intervening stages. The liver stiffness is also impacted by the severity of inflammation, vascular congestion, or cholestasis [77]. There is also an association between ALT levels and liver stiffness [78]. Ingestion of a meal increases liver blood flow and stiffness as well and a standardized set of best practices for the use of this technology has been published [79].

ARFI has the advantage of being available along with liver images and is part of ultrasound imaging platforms. Shear wave elastography is a recent innovation that has also been recently validated in terms of its reproducibility and repeatability etc. [80]. In a recent head-to-head comparison, these methods were by and large comparable [81].

MRE can be used on a 2-dimensional (2D) or 3-dimensional (3D) platform. The majority of the literature is, however, based on 2D MRE. Several studies have reported that it can accurately define the fibrosis stage with area under the curve (AUC) of 0.9 for cirrhosis, and is modestly but statistically significantly superior to FibroScan and FIB-4 indices [82, 83]. It is also impacted by other factors that determine liver stiffness. Also, up to 10% of patients with NASH are unable to undergo MRE due to claustrophobia, excess body weight and waist circumference, inability to lay down for the procedure, metal in their body, etc. MRE is also substantially more expensive in the Western world than ultrasound-based methods. Importantly, the test–retest variability for 2D MRE is 15–20% [84]. 3D MRE carries the promise of overcoming several limitations of 2D MRE and is an area of active investigation.

Another MR-based technology is the Multiscan™, which was developed at Oxford University. It provides a combined measure of inflammation and fibrosis in the form of a liver inflammation and fibrosis (LIF) score based on corrected T1 images (cT1) values [85]. In that study, cT1 images were significantly different between all fibrosis stages, except between mild and moderate fibrosis. In addition, rapid spectroscopy correlated well with other steatosis scores ($r^2 = 0.89$; $p < 0.0001$) [87].

5 Tests of Fibrogenesis Using Incorporation of Label into Fibrogenic Products

It has been known for many years that the incorporation of deuterium can be leveraged to determine the rates of formation of various substances. This was used in an exploratory study where deuterium was administered for several weeks followed by measurement of its incorporation into lumican, a matrix protein that is also released into circulation [86]. These indicated that rates of deuterium incorporation into lumican correlated with fibrosis stage in a cross-sectional study. This is, however, a cumbersome process and is not suitable for routine clinical use, although it may be valuable for the assessment of fibrogenesis in research settings.

6 Role of Non-invasive Markers of Liver Fibrosis in Clinical Research

The noninvasive biomarkers of liver fibrosis have become the focus of research attention over the past decade. In general, most biomarkers are more accurate for advanced-stage liver disease. In order to increase diagnostic accuracy, studies have recently begun to evaluate combinations of serum biomarkers and imaging [132]. Several biomarkers have already been established for the management of HCV; however, most of them are still being evaluated for their efficacy in NAFLD, hepatitis B virus, and alcoholic liver disease [133, 134]. Moreover, it has been suggested that these tests may be able to reduce the need for liver biopsy by serving as pre-screening tools to rule out advanced liver disease [135]. There are concerns regarding the methodology of the studies that were conducted; for example, they were often small, had spectrum bias which limits applicability, and a majority did not replicate the development and validation cohorts. The definition of AUROC is not absolute, and depending on it results in missing the clinical context. Therefore, the clinical question determines the metric of accuracy of the optimal diagnostic test.

7 Conclusions

Liver biopsy is not practical for use to screen for NAFLD due to several disadvantages related to cost, availability, safety, and sampling errors. The need for non-invasive tests is growing, therefore many serum biomarkers have

emerged. Recent years have witnessed the development of various non-invasive methods to assess liver fibrosis and guide the clinical management of NAFLD/NASH. This is important, especially upon follow-up of disease progression using serial measurements and tracking response to treatment. In addition, scoring systems such as FIB-4 and APRI are readily available and easy to use. Imaging modalities that assess liver stiffness, such as FibroScan, are becoming more commonly used and have gained popularity in assessing the degree of liver fibrosis given their accuracy and reproducibility. However, to date, non-invasive tests remain challenged by the staging of fibrosis in its early phases. Thus, their performance depends on each individual case, underlying etiology of NAFLD, and stages of the disease. The extensive research is focusing on finding solutions to those limitations and improving the accuracy of these tests so they can one day become a replacement for liver biopsy.

Compliance with Ethical Standards

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