Noninvasive Diagnostic Options for Assessing Fibrosis and Liver Function in NAFLD/NASH

Joseph Sebastian[1], Mark H DeLegge[2]* and John D Cochran[3]

[1]*Director Scientific Affairs, Infectious and Liver Diseases, Q2 Solutions, Morrisville, NC, USA*
[2]*Senior Medical Director GI Center Excellence, IQVIA, Durham, NC, USA*
[3]*Chief Pathologist, Q2 Solutions, Morrisville, NC, USA*

*Corresponding Author: Mark H DeLegge, Senior Medical Director GI Center Excellence, IQVIA, Durham, NC, USA.

Abstract

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are an increasingly common cause of liver disease globally. Currently the gold standard for making the diagnosis and tracking progression of the disease over time is liver biopsy. There have been many publications and abundant clinical experience regarding the use of clinical scoring systems and serum and imaging biomarkers for the diagnosis of NASH. To date, none of these non-invasive tests have been accepted to replace liver biopsy However, from a standpoint of evaluating disease outcome over time it has been recognized that an individual's stage of liver fibrosis has direct correlation with associated liver outcomes such as progression to liver failure. There have been many efforts focused on non-invasively determining a patient's liver fibrosis stage without the need for liver biopsy. These fibrosis biomarkers can be divided into direct and indirect serum biomarkers and imaging technologies with a focus on transient elastography and MRI-based technologies. However, neither liver biopsy nor non-invasive biomarkers of fibrosis can determine actual liver function. Assessing liver function can be important in determining when a patient moves from compensated cirrhosis to decompensated cirrhosis. Functional liver function tests can be separated into 2 categories: serum metabolite measurements after oral or intravenous substance administration and breath tests using stable radioisotopes. These functional tests currently have limited commercial availability.

Keywords: Non-Alcoholic Fatty Liver Disease (NAFLD); Non-Alcoholic Steatohepatitis (NASH); Fibrosis

Introduction

Non-alcoholic fatty liver disease (NAFLD)

Hepatic disease comprises a wide range of complex conditions. Liver diseases are extremely costly in terms of human suffering, physician and hospital visits, premature loss of life and productivity. Based on 2019 data, liver diseases account for approximately 2 million deaths per year worldwide [1]. Cirrhosis is currently the 11th most common cause of death and liver cancer is the 16th leading cause of death; combined, they account for 3.5% of all deaths globally [2].

Worldwide, approximately 2 billion adults are obese or overweight and over 400 million have diabetes; both of which are risk factors for non-alcoholic fatty liver diseases (NAFLD) [1,3,4]. Before developing cirrhosis, or late stage scarring of the liver tissue, patients are

often diagnosed with a form of progressive liver disease called NAFLD or more specifically non-alcoholic steatohepatitis (NASH) [5]. Non-alcoholic fatty liver disease is the presence of excessive fat in patients' liver cells (steatosis) not associated with alcohol abuse. This accumulation of fat, in some patients, leads to a chronic inflammatory process with the ultimate formation of fibrosis and is known as NASH. While patients with NASH are at significantly increased risk of advancing fibrosis/cirrhosis, many will not develop progressive disease. A small percent of NAFLD patients can also develop progressive disease in the apparent absence of NASH. It is now widely recognized that fibrosis is the most important histological feature associated with overall survival and liver-related complications. Ideally, testing all patients with NAFLD for the presence of NASH will allow clinicians to determine the needs for treatment and ongoing monitoring [6].

This disease is currently the largest public threat from the family of chronic liver diseases, affecting approximately 25% of the global population [6]. It is estimated to impact about 30% of the US population, or 85 million people [5,7]. Approximately 15 - 20% of patients with NAFLD progress to NASH. Patients with NASH often develop more serious liver conditions, such as fibrosis/cirrhosis of the liver and hepatocellular carcinoma (HCC). NASH is the hepatic manifestation of metabolic syndrome, characterized by insulin resistance, obesity, and hyperlipidemia [8].

To understand whether NAFLD patients are progressing, stable, or improving, they should be monitored for progression to NASH, as advancing disease is associated with hepatocyte dysfunction [9]. The key pathogenic driver in NASH is the development of liver fibrosis as progressive scarring and disruption of the hepatic tissue can culminate in dysfunction, liver failure or liver cancer. With intervention, fibrosis may be stabilized, or even reversed and liver outcomes improved.

Determining the degree of chronic liver disease

The primary diagnostic element often able to correlate to patient clinical outcomes is the level of scarring in the liver. This continuum of liver scarring starts as liver inflammation of various etiologies. In response to inflammation, regenerating hepatocytes surround the fibrotic tissue and reorganize the liver blood flow in attempt to reestablish homeostasis. This process in turn substantially disrupts liver function. This is the next step on the continuum from normal hepatic tissue to cirrhosis.

Liver biopsy is often used to identify both NASH and the degree of fibrosis. Patients with advanced fibrosis or cirrhosis have a significantly higher risk of adverse outcomes and can benefit from expedient intervention and monitoring. However, liver biopsy is invasive, carries risk and requires access to a site where the procedure can be performed. Limitations or factors affecting accuracies of liver biopsy include sampling error, quality/size of sample and interobserver variation in histologic assessment. As a result, there is increasing demand for non-invasive alternatives to assess for liver fibrosis in NAFLD patients. Currently, the use of serum biomarkers and imaging are growing in the diagnostic space. Blood-based, non-invasive testing methods for liver fibrosis are particularly appealing as they require only a routine blood sample and support high-throughput testing. This would satisfy the requirements of a screening diagnostic test assuming pricing was reasonable.

Static blood tests

Routine blood tests such as serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin and the International Normalized Ratio (INR) are widely used for clinical routine assessment of liver function. However, these are not useful in the diagnosis of NAFLD or NASH or to accurately determine the presence or degree of fibrosis. Currently, NASH can only be diagnosed by a liver biopsy; however, there is a high incidence of complications and variability in diagnosis depending on the evaluating pathologist. There is no validated, non-invasive test available to differentiate NAFLD from NASH. Monitoring patients for the presence of advanced fibrosis (≥ F2) is critical as these individuals may benefit from multidisciplinary treatment of metabolic syndrome including weight loss,

exercise and glucose control. Once a pharmacologic treatment for NASH is available these same non-invasive biomarkers may be used to determine who needs to be treated and for how long they require treatment.

Invasive liver tests

Liver biopsy

Histological examination of liver tissue is considered as the reference standard for the diagnosis and monitoring of liver fibrosis and cirrhosis [10]. Histological findings for NASH provide a spectrum of information including liver architecture, presence and extent of steatosis, grade of necroinflammation and the extent of liver fibrosis. In cases of unexplained liver functional abnormalities biopsy can provide a diagnosis. This level of information is not yet available by any current, regulatory approved non-invasive test for NAFLD and more specifically, NASH.

The sample taken by biopsy needle is a volume of less than 0.01% of the total liver volume; the sampling variability could potentially misclassify the extent of fibrosis [10]. Even when experienced liver histopathologists are involved, histological staging is prone to intra- and interobserver variability [10]. The percentage of biopsy concordance with reference evaluations, according to Fatty Liver Inhibition of Progression (FLIP) consortium publications, was between 42% and 77% [11].

As there is currently no available pharmacologic treatment for NASH, there has been a paucity of liver biopsies performed in at-risk patients as diet, exercise and glucose control can be advised for all at-risk patients, irrespective of the diagnosis of NASH. There is a low patient acceptance of liver biopsy for the diagnosis of NASH in the current era of non-invasive technologies for disease detection. The biopsy procedure can be painful, time consuming and has associated risk. It often requires a facility with an ultrasound machine, sometimes an anesthesiologist and a specialist in biopsies. The main risk of the procedure is clinically significant bleeding (1.1 - 1.6%), which can be potentially fatal [12]. Biopsies of an adequate length (25 mm) are not always obtainable with one needle pass in a percutaneous biopsy and multiple attempts at tissue sampling may be required.

Although the exact diagnosis of NASH requires a liver biopsy, this procedure alone does not provide all pertinent information needed for a complete clinical diagnosis [13,14]. NASH histologically is characterized by parenchymal injury, including macro-vesicular steatosis, ballooning degeneration, Mallory-Denk bodies and inflammation in hepatic lobes. However, a biopsy cannot quantify the functional reserve of hepatocyte mass or the degree of impairment in the blood perfusion of hepatocytes.

Hepatic venous pressure gradient

Hepatic venous pressure gradient (HVPG) has been proposed as a surrogate marker for the evaluation of chronic liver disease. It is a measurement meant to represent portal venous pressure and therefore serve as a marker of the severity of cirrhosis. HVPG measurement is done under local anesthesia in an interventional radiology suite; a venous introducer is placed in the right internal jugular vein by the Seldinger technique [15]. A 7F balloon-tipped catheter is guided into the right hepatic vein for measurement of wedged and free hepatic venous pressure. The HVPG calculates the difference between wedged hepatic venous pressure and free hepatic venous pressure. Usually, these measurements will be performed in triplicate and a permanent tracing is recorded on a multichannel recorder. A HVPG > 10 mm Hg identifies clinically significant portal hypertension. This wedge pressure reflects the portal vein pressure, which in turn is increased if the liver resistance is increased as proportional to liver fibrosis. The HVPG is well correlated to clinical scoring systems of liver status such as MELD and Childs-Pugh scores [16]. The test is expensive and not available at many medical centers.

Non-invasive liver tests

During the last few years there has been an explosion in the development and attempted validation of non-invasive liver tests [17]. These tests hope to replace liver biopsy in clinical practice for the staging of fibrosis and follow-up of patients with established chronic liver disease. The non-invasive liver tests (NILTs) can be broadly divided into three categories: simple or indirect serum markers, direct serum markers and imaging modalities. These modalities can be used alone or together with other tests. Some have been combined in patented commercial algorithms which may improve the diagnostic accuracy of these tests. Examples of such tests are ELF™, FibroTest™, FIB4, APRI, NIS4, FIBROSpect®, Fibroindex and Fibrometer™. Many of these NILT have been utilized in NASH clinical trials as either a secondary or exploratory outcome.

Indirect serum markers

Indirect serum markers, or class II biomarkers, consist of the combination of routine biochemical or hematological tests, such as transaminases, platelet count and albumin and patient demographics that are associated with fibrosis, such as age or the presence of diabetes. Table 1 reviews the sensitivity and specificity of these biomarkers.

Test	Components	Cutoff	Sensitivity	Specificity	NPV
FIB-4	ALT, AST, age, PLT	1.03 - 2.67	0.84	0.74	0.98
NFS	Age, BMI, T2D, AST, ALT, PLT, Albumin	0.676 - 1.455	0.8	0.66	0.98
ELF	Hyaluronic acid, PIINP, TIMP_1	9.5	0.81	0.9	0.99
NIS4	MiR-34a, α-2 Macroglobulin, YKL-40, HemoglobinA1c	0-1	0.82	0.72	0.99
FibroTest	α-2 Macroglobulin, Haptoglobin, Apo-A1, Bilirubin, GGT, γ-globulin	0.3, 0.7	0.88	0.73	0.99
FibroMeter	Hyaluronate, α-2-macroglobulin, prothrombin index (or INR), AST, urea and platelets	0.31	.0.8	0.8	0.86
FIBROSpect	Hyaluronic acid (HA), tissue inhibitor of metalloprotein-ase-1 (TIMP-1) and alpha2-macroglobulin (A2M)	n/a	0.81	0.71	0.3

Table 1: Overview of non-invasive biomarkers for NAFLD.

NAFLD fibrosis score (NFS)

This scoring system has six variables: age, hyperglycemia, body mass index, platelet count, albumin, and AST/ALT. The NFS has been validated in multiple studies; a recent meta-analysis revealed an AUROC of 0.85 (95% CI, 0.81 - 0.90) [18]. With these tests many patients typically fall in the indeterminate range of fibrosis and will need either further sequential, non-invasive testing or an additional invasive testing to determine the degree of fibrosis.

FibroTest

FibroTest™ (FT), ActiTest™ (AT) (Biopredictive, Paris, France), known as FibroSURE® in the US (LabCorp, Burlington, NC, USA) is one of the most widely used NILTS. FT includes 2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin, and GGT and is adjusted for age and gender. Actitest (AT) includes the same 5 components as FT plus serum transaminases [19]. A study of FT and AT analyzed data from 484 patients; pooled data demonstrated an AUROC of 0.83 (0.78 - 0.88) for FT and 0.84 (0.79 - .088) for AT. The latest version of these tests eliminates the need to add body mass index (BMI) to the algorithm [20]. These related tests were the first hepatic fibrosis indirect serum biomarkers which received wide range acceptance from the medical community with the most cited references [21].

FIBROSpect

FIBROSpect® (Prometheus Laboratories, San Diego, CA, USA) NASH is a laboratory-developed test that aids in in the detection, staging, and monitoring of liver fibrosis in nonalcoholic steatohepatitis patients. The markers used in the calculation of fibrosis are tissue inhibitor metalloprotease inhibitor 1 (TIMP1), hyaluronic acid and alpha-2 macroglobulin [22]. In a cohort of patients with biopsy-proven NASH (n = 396), results of FIBROSpect NASH were validated against histologic fibrosis stage (as defined by NASH CRN criteria) using serum acquired on the same day as the liver biopsy [21]. The FIBROSpect NASH test was further validated in a combined independent cohort (n = 640) of patients with biopsy-proven NASH obtained from two geographically distinct locations [21] with an AUROC of 0.86 (95% CI: 0.82 - 0.89). Sensitivity and specificity for identifying NASH patients with advanced hepatic fibrosis was 80% (95% CI: 72 - 86%) and 76% (95% CI: 72 - 79%), respectively.

Fiber index (FIB4)

The FIB-4 scoring system uses a combination of patient age, platelet count, AST and ALT - all tests available to the primary care physician (PCP) [23]. The scoring system notes a score < 1.45 has a negative predictive value of over 90% for advanced liver fibrosis from multiple etiologies and a value greater than 3.25 predicts an advanced fibrosis score [23]. In the United Kingdom this score is being increasingly used to stratify Fibrosis in NAFLD patients (Figure 1) [24]. In an analysis of a PCP practice, many patients have a value below the lower cut off value (-1.455), hence advanced fibrosis could be excluded with very high accuracy (negative predictive value of 93%) [24].
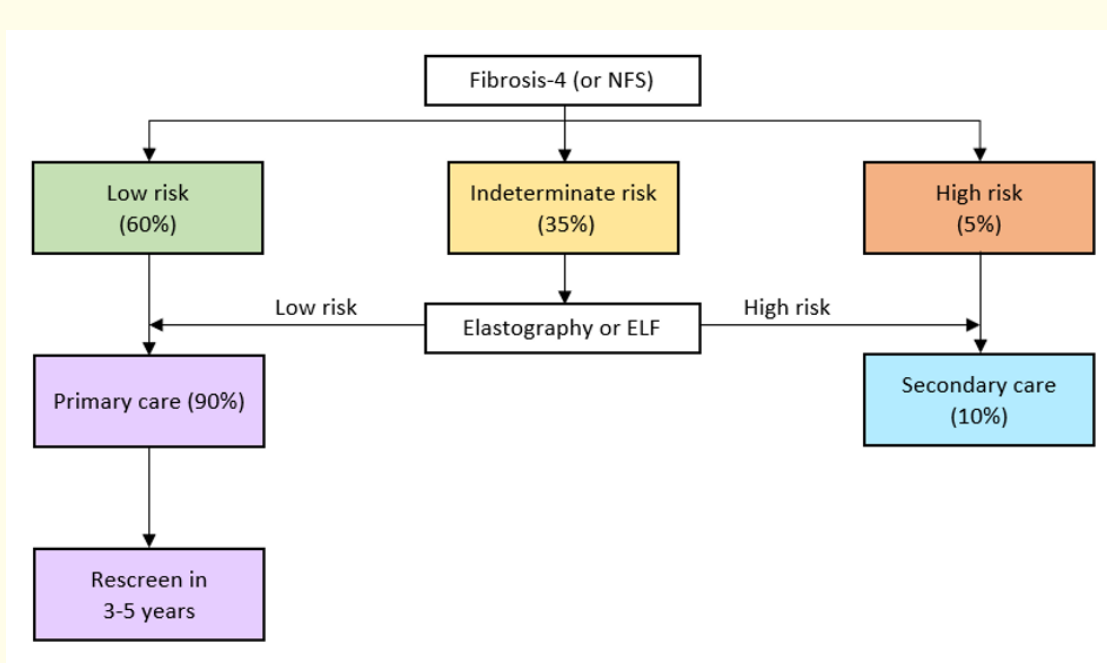

Figure 1: Stratification of care in patients at-risk for NASH.

FibroMeter

FibroMeter[TM] (ARUP Laboratories, Salt Lake City, UT, USA) is a family of liver fibrosis tests based on blood markers. FibroMeter was one of the first non-invasive tests developed for assessing liver fibrosis in 1997 [25]. Initially, a specific version was developed for NAFLD, the FibroMeter[NAFLD] which included routine blood biomarkers [26]. However, FibroMeter[V2G], which includes direct fibrosis markers, has been shown to be superior in NAFLD [27,28]. The blood markers of FibroMeter[V2G] are: hyaluronate, alpha2-macroglobulin, prothrombin index (or INR), AST, urea and platelets. FibroMeter[V2G] has been validated in a population of 938 patients with biopsy-proven NAFLD where it was the most accurate blood test for the diagnosis of advanced fibrosis [29]. FibroMeter[VCTE] combines vibration controlled transient elastography (VCTE) (Fibroscan® - Echosense, Waltham, MA) results and FibroMeter biomarkers. FibroMeter[VCTE] outperformed VCTE alone and other blood tests (AUROC: 0.866, p ≤ 0.005) for the diagnosis of advanced fibrosis in the same population [29]. FibroMeter[V2G] and FibroMeter[VCTE] have been validated by independent teams in NAFLD [30].

NIS4

NIS4 (Genfit, Loos, FR) is a biomarker-based test that assigns a continuous score (0 to 1.0) based on the quantification of four circulating biomarkers: MiR-34a, Alpha2-macroglobulin, YKL-40 and Hemoglobin A1c [31]. The data from a NASH clinical trial (GOLDEN trial) was used to identify and monitor NASH and Fibrosis. This biomarker-based test was evaluated using biomarkers from the GOLDEN trial and subsequently validated using a separate NASH trial (RESOLVE trial) [30]. In these trials NASH patients At-Risk-of-Cirrhosis (ARC) were defined by NAS (non-alcoholic fatty liver disease score) ≥ 4 and F ≥ 2 and patients Not-At-Risk-of-Cirrhosis (NARC) by NAS < 4 and/or F < 2. The training cohort (GOLDEN or G) was comprised of 220 patients (ARC/NARC = 95/125). The validation cohort (RESOLVE or R) was comprised of 467 patients (ARC/NARC = 255/212). Diagnostic performances (ARC vs NARC) in G and R were compared (AUROC, sensitivity, specificity). The combined merged cohort (M) with 687 patients was used for optimization of coefficients, assessment of relations with NAS and a Fibrosis score (F) and comparison with existing score detection of ARC. After optimization in these merged studies, AUROC was shown to be 0.82 (0.78 - 0.85) [31]. At optimal cutoff for M, sensitivity and specificity were both 76%. Furthermore, NIS4 gradually increased with NAS and fibrosis score and was more potent than existing scores for detection of ARC vs NARC. These studies validate NIS4 clinical performance for detection of ARC in a large population of patients prospectively screened for suspicion of progressive NASH at many hepatology centers in several different countries [31]. The high diagnostic accuracy was established irrespective of the age, gender, presence of diabetes, metabolic syndrome or obesity.

Direct serum non-invasive tests

Direct serum non-invasive tests or class I biomarkers are intended to detect extracellular matrix turnover and/or fibrogenic cell changes in liver. The most common markers used in current assays involve measuring products of extracellular matrix synthesis or degradation and the enzymes that regulate their production or modification, such as hyaluronic acid, serum collagenases and their inhibitors and profibrogenic cytokines.

ELF test

Liver fibrosis is biochemically complex but orchestrated primarily by activated hepatic stellate cells (HSCs). Activated HSCs produce components of the extracellular matrix (ECM). The ECM includes an array of proteins involved in scar formation, including fibronectin, laminin, collagens, hyaluronic acid (HA) and proteoglycans. Collagen types I, III, IV and V are prominently expressed within the liver [32]. HA is an essential component of the ECM and is produced primarily by HSC [32,33]. The accumulation of deposited ECM progressively replaces the normal liver parenchyma, producing damage and scar tissue and ultimately disrupting hepatic architecture and function. Fibrosis of the liver is a largely bidirectional process [34,35]. Both fibrosis and repair mechanisms have been linked to ECM-related

pathways. Regression and repair are associated with upregulation of matrix metalloproteinases (MMPs), which are a family of zinc-dependent endopeptidases capable of degrading ECM deposition and therefore central to healing. Levels of MMPs are subject to inhibition by tissue inhibitors of metalloproteinases (TIMPs), a family of at least four proteins (TIMP 1-4) that bind MMPs. TIMP-1 overexpression hinders degradation and clearance of the fibrotic matrix, leading to increased levels of interstitial ECM and progressive fibrosis [36,37].

The Enhanced Liver Fibrosis (ELF™) (Siemens, Munich, DE) test is a noninvasive blood (serum) lab test designed to assess levels of three major components directly involved in liver matrix metabolism: hyaluronic acid (HA), procollagen III amino terminal peptide (PIIINP), and tissue inhibitor of matrix metalloproteinase 1 (TIMP-1). The analytes are automatically measured, and the software calculates and reports a unitless numeric score. Elevated ELF scores are linked to both biopsy-proven fibrosis/cirrhosis and prognosis for clinically significant outcomes. ELF has been widely investigated across multiple forms of CLD, with the seminal publication occurring in 2004 [38]. Subsequently, dozens of studies and publications now support the clinical utility of ELF, including diagnostic and prognostic performance. The ELF score has been well-validated against biopsy-proven fibrosis across a range of chronic liver diseases (CLD) in both adult and pediatric populations [38,39]. Additionally, outcome data supports a strong prognostic performance for ELF across multiple forms of CLD, including NAFLD/NASH [39]. The potential of the ELF test to aid discrimination of higher-risk patients may be especially useful in a primary care setting when determining need for specialist referral [40].

The ELF test is available on the following immunoassay laboratory instruments: Atellica IM® Analyzers and ADVIA Centaur® Systems, broadly available worldwide in many labs offering routine lab testing. By testing for direct markers associated with both ECM deposition and repair, the ELF test provides a direct measure for the assessment of fibrotic activity [40]. AUROC analysis for the diagnostic threshold (cutoff) was 0.88 for the detection of significant fibrosis, 0.87 for severe fibrosis, and 0.88 for cirrhosis. Pooled sensitivity for the performance of the ELF test in the assessment of significant fibrosis was 83% and pooled specificity 73% [41]. For the prediction of severe fibrosis, the pooled sensitivity value was 78% and pooled specificity 76%. Pooled sensitivity for the prediction of cirrhosis was 80% and pooled specificity 71% [41].

Pro-C3 testing

In the healthy human liver, fibril-forming types I and III collagens are integrated into the ECM after removal of the N- and C-terminal propeptides. Removal of the N-terminal propeptide is sometimes incomplete leaving it attached to the collagen helix resulting in thin fibrils with abnormal cross-links and thereby making it susceptible to rapid metabolic turnover [42,43]. Thus, a conventional N-terminal propeptide of type III collagen (PIIINP) epitope can be a marker of both fibrogenesis and fibrolysis. In the newly designed Pro-C3 (Nordic Bioscience Herlev, DK) enzyme-linked immunosorbent assay (ELISA), by targeting the N-protease cleavage site of PIIINP (Pro-C3) only formation, but not degradation of type III collagen is identified [43]. Thus, Pro-C3 maintains specificity towards the cleaved PIIINP and is thereby much more specific for type III procollagen synthesis and tissue deposition than conventional PIIINP assays. Recent publications established the role of PRO-C3 (a marker of type III collagen formation) as a biomarker for advanced fibrosis in NAFLD. A PRO-C3 based fibrosis algorithm called "ADAPT" was developed that includes age, presence of diabetes, PRO-C3 itself, and platelet count. PRO-C3 was found to increase with fibrosis stage (rho 0.50 p < 0.0001) and was independently associated with advanced fibrosis (OR = 1.05, 95% CI 1.02 - 1.08, p = 0.003) [43]. ADAPT demonstrated areas under the receiver operating characteristics curve (AUROC) of 0.86 (95% CI 0.79 to 0.91) in the derivation and 0.87 in the validation cohort (95% CI 0.83 to 0.91) for advanced fibrosis.

Limitations of non-invasive marker-based tests

It is important to note that the low level of adoption for non-invasive tests may be due to the histologic misclassification rate (percentage of incorrect staging of fibrosis) from liver biopsy which is used as the gold standard. It is possible that the false positive or false negative rate of such a test is a fault of the biopsy rather than the test itself. The reality is, despite the inherent limitations of biopsy, almost all non-invasive fibrosis markers and imaging techniques have been developed and calibrated with direct reference to a set of liver biopsies.

As a result of the need for liver biopsy for diagnosis and the lack of an accepted non-invasive biomarker, NASH is underdiagnosed and understudied. From a drug development perspective, the United States Food and Drug Administration has required pharmaceutical companies to uniformly apply NASH resolution or fibrosis improvement as the primary endpoint of phase IIb and III trials. Moreover, histologically determined fibrosis in NASH is a discontinuous variable and requires years to change, thus prolonging therapeutic trials, especially those interested in looking at the long-term efficacy of a proposed new drug. With that, imaging modalities have attempted to fill the gap and provide a technology for assessing the overall liver fibrosis content.

Innovative imaging modalities

New imaging techniques offer better sensitivity and specificity than conventional techniques, such as ultrasound, computed tomography (CT) and standard magnetic resonance imaging (MRI). A conventional MRI can only identify cirrhosis based on the imaging findings of coarse echo-texture, collaterals suggestive of portal hypertension and nodularity. These new modalities measure liver elasticity or liver stiffness based on modifications of ultrasound or magnetic resonance (MR) techniques.

The most widely used imaging modality is vibration controlled transient elastography also referred to as Fibroscan® (Echosens Paris, FR) and Fibrotouch® (Hisky Medical Jiangsu. China). Other new modalities include acoustic radiation force impulse (ARFI), Liver Multiparametric Scan (LMS) and MR elastography (MRE).

Transient elastography (TE)

This technique uses an ultrasound transducer to induce an elastic shear wave that propagates within the liver that is directly related to the tissue stiffness (the harder the tissue, the faster the shear propagates). Results are a good indication of the amount of liver stiffness in the liver and are expressed in kilopascals (kPa) and correspond to the median value of 10 validated measurements ranging from 2.5 to 75 kPa, with 5.5 kPa reported to define normal [44]. There is a correlation between the liver stiffness measurement and the degree of liver fibrosis. Moreover, TE is painless, can be performed at the physician's office and is rapid to deploy (< 5 minutes) and thus highly acceptable to patients. A considerable amount of data is evolving now to support the value of TE as a recruitment enrichment technique to successfully decrease screen failure rates in NASH clinical trials. Another measurement provided by TE, specifically designed for detecting the degree of steatosis, is controlled attenuation parameter or CAP and is measured in dB/m (range between 100 and 400). By using of the combination of CAP, liver stiffness and other non-invasive biomarkers the right NASH patients for liver biopsy can be identified. For, investigators in clinical trials, this can further decrease the screen failure rates to as low as 25% [45].

Acoustic radiation force impulse (ARFI)

ARFI allows the evaluation of liver stiffness in a region of interest (ROI) by using mechanical excitation of tissue with the use of short-duration (≈262 μs) acoustic pulses while performing a real-time B-mode conventional hepatic ultrasound [46]. Results are expressed in m/s. Although the volume of liver explored is smaller than that for TE (10 mm long × 6 mm wide), a critical advantage is the possibility to choose the representative area of interest to test by using an ultrasound image to avoid potentially conflicting structures such as large vessels, cysts, tumors and ribs. Furthermore, it can be easily incorporated into a modified ultrasound machine and hence, does not require an acquisition of a separate device [47].

Liver multiparametric scan (LMS)

Recently a new multiparametric MRI (Perspectrum Diagnostics Oxford, UK) technology has been introduced that is based on 3 different components: T1 mapping for fibrosis/inflammation, T2 mapping for liver iron quantification and proton magnetic resonance spectroscopy (1H-MRS) for liver fat quantification [48]. This technology has shown a strong correlation with NASH histological parameters in mixed

patient groups in early studies [49]. In a recent comparative evaluation of different imaging modalities for NAFLD, one investigator concluded that LMS was considered best in its ability to identify NASH. However, further validation will be required. It does require a radiologic center visit and can be cost prohibitive.

Magnetic resonance elastography (MRE)

Magnetic resonance elastography (Resoundant Rochester, MN USA) uses a modified phase-contrast method to evaluate the propagation of the shear waves within the liver [50]. It is a very promising technique for evaluating liver fibrosis but is not yet widely available outside of tertiary care centers and can be cost prohibitive. It requires a visit to a radiologist. A recent meta-analysis demonstrated that MRE has the highest diagnostic accuracy for staging fibrosis in NAFLD patients [51].

Limitations of innovative imaging modalities

These new imaging techniques are not without their limitations. They are unreliable in acute liver inflammation, particularly during ALT flares. They are influenced by food intake and it is suggested that imaging should be performed after at least a 3 hour fast to ensure accuracy of fibrosis assessment. Lastly, they cannot reliably predict cirrhosis without a biopsy confirmation as an elevated liver stiffness value cannot accurately differentiate between hepatic congestion and hepatic fibrosis.

Any imaging technique has the likelihood of technical failure for a variety of reasons. Recently a large review of 13,369 TE examinations over 5 years demonstrated LSM failure in 3.1% and unreliable LSM in 15.8% in the same patient population [52]. Both were associated with two main factors: elevated body mass index (BMI) > 30 kg/m^2 and operator experience of less than 500 examinations. One of the difficulties in using TE in routine clinical practice is the variability of optimal cut-off levels for the diagnosis of fibrosis and cirrhosis in different etiologies of liver disease as seen in a meta-analysis of 40 studies evaluating the diagnostic accuracy of TE in various chronic liver disease [51]. In addition, there are concerns about the accuracy of TE in patients with a BMI > 35.

The quest for the "functional liver" test

Assessment of liver function throughout the course of diagnosis and treatment remains imprecise. Liver biopsy cannot quantify functional hepatocyte biomass or decreased hepatocyte perfusion caused by porto-systemic shunting. Imaging and serum biomarkers described in this paper do not measure liver function. Intuitively it is assumed that the greater the level of liver fibrosis, the less the overall liver function. However, there is not a 1:1 correlation. A recent paper has concluded that the liver remains the only organ without a validated and widely accepted functional test [53]. In fact, most "liver functional tests" used in clinical practice do not accurately deliver truly functional information. These tests include serum liver enzymes, synthetic tests, as well as the popular clinical scoring systems we use to "estimate" the functional capacity of the liver such as the Child-Turcotte-Pugh (CTP) and the model for end-stage liver disease (MELD) score. These scoring systems are 'rough" predictors of the function of the liver, especially in the earlier stages of liver disease.

Functional liver function tests can be separated into 2 categories: serum metabolite measurements after oral or intravenous substance administration and breath tests using stable radioisotopes.

Serum metabolite measurements

MEGX

Lidocaine is converted into monoethylglycinexylodide (MEGX) by cytochrome P-4503A4 in the liver. This metabolite rapidly shows up in the serum in a steady state after hepatic metabolism. In one study MEGX serum concentrations were obtained 15 minutes after

intravenous administration of a single dose of lidocaine in 24 adults with chronic hepatitis, 47 patients with cirrhosis and 26 normal controls. The MEGX concentrations in controls was higher than those in hepatitis which was higher than those with cirrhosis (p < 0.05). In addition, the serum MEGX levels were inversely proportional to Child's Pugh's score and the prothrombin time. A MEGX concentration of below 54 ng/ml was an indicator of hepatic dysfunction with a diagnostic sensitivity for detecting hepatic disorder of 84.5%, a specificity of 88.5% and an accuracy 85.6% [54].

Indocyanine green

Indocyanine green is a dye that has been used to measure hepatic blood flow, and indirectly, hepatic function. Techniques have been developed to measure hepatic indocyanine clearance. The indocyanine green clearance test (clearance rate (K) and retention rate at 15 minutes (R15)) is believed to be a sensitive indicator to evaluate liver function. In a prospective study, 52 patients with liver disease classified into Child-Pugh class A (8), B (14) and C (30) were evaluated. The indocyanine green clearance test (K value and R15) was performed and the MELD scores of patients were calculated. As the Child-Pugh score gradually deteriorated, the K value decreased, while R15 and MELD score increased. There were significant statistical differences in K value, R15 and MELD score in patients with different Child-Pugh classifications. A negative correlation was observed between K value and MELD score (r = -0.892, P < 0.05), while a positive correlation was observed between R15 and MELD score (r = 0.804, P < 0.05) [55].

Neither MEGX nor indocyanine green have received extensive evaluation for determining liver function in a NASH population.

Breath tests

Breath tests using radiolabeled carbon metabolites have been used for assessment of liver function. These are generally performed with carbon-13. Some of these are commercially available. There use amongst hepatologist is sporadic for the assessment of liver function with an emphasis on identifying which patient will move from compensated cirrhosis to decompensated cirrhosis

Methacetin

Methacetin is a substance that undergoes 1st pass clearance in the liver and is excreted in the urine.13C-methacetin breath test has been studied in chronic liver disease. It was able to differentiate non-cirrhotic patients from patients with Child's class A cirrhosis with 95% sensitivity and 97% specificity [56].

^{13}C-phenylalanne, ^{13}C-galactose, ^{13}C-aminopyrine

^{13}C-phenylalanine and ^{13}C-galactose are radiolabeled substances that can measure liver function by assessing enzymes that are in the cytosol of the hepatocyte (phenylalanine hydroxylase and galactose kinase). Aminopyrine is an alternative compound that is metabolized by hydroxylation in the liver. Sixty patients with chronic liver disease of diverse etiologies received a ^{13}C-galactose and ^{13}C-aminopyrine breath test. The combined assessment of the ^{13}C-galactose and ^{13}C-aminopyrine breath test increased the diagnostic accuracy (80% positive predictive value) of either test alone for detecting chronic liver dysfunction and reached 92.5% specificity and 100% sensitivity for the diagnosis of cirrhosis [57]. A separate study evaluated the use of ^{13}C-methacetin breath test and the ^{13}C- phenylalanine breath test in 48 patients with chronic liver disease and 48 patients with normal health volunteers. Correlation between ^{13}C-Phenylalanine Breath Test and ^{13}C-Methacetin Breath Test was 0.63, p < 0.001. If both tests were abnormal, the sensitivity for the diagnosis of hepatic dysfunction was high (98%), although the specificity decreased to 60% [58].

^{13}C-caffeine

Caffeine is a substance with known pharmacokinetics and is almost exclusively metabolized in the liver by demethylation by the cytochrome P450 system. ^{13}C-caffeine breath test was performed in 25 healthy controls; 20 subjects with noncirrhotic, chronic hepatitis B or C; and 20 subjects with cirrhosis. Cirrhotic patients were characterized by significantly reduced CBT values with controls (p < .001) and hepatitis patients (p < .04). There was a significant inverse relationship between the CBT and Child-Pugh score (p < .002) [59].

Breath tests in NAFLD and NASH

Some of these hepatic function breath tests have been evaluated in the NASH patient population. ^{13}C-methacetin breath test has been evaluated in 64 patients with histologically proven NAFLD (ranging from simple steatosis to severe steatohepatitis) and in 20 healthy controls. ^{13}C-methacetin breath testing identified patients with histologically proven NASH, with an AUROC of 0.824, 95% CI (0.723 - 0.926), a sensitivity of 95% and a specificity of 74% [60]. In a separate study, thirty-six patients with histologically proven NAFLD (NAFL:16, NASH:20) received ^{13}C-aminopyrine breath test and 13C-galactose breath test. 13C-ABT results correlated inversely with activity grade (r = −0.650, P = 0.001), NAFLD activity score (r = −0.473, P = 0.026) and fibrosis stage (r = −0.719, P = 0.001). In contrast, there was no significant association between 13C-galactose breath test results and any patient characteristic [61].

^{13}C caffeine breath test was investigated in 48 patients with NAFLD and healthy controls. The results were compared to histological liver data in the NAFLD patients. Patients with simple steatosis on histology had similar ^{13}C caffeine breath test values to controls (p = 1.0). However, ^{13}C caffeine breath test was significantly reduced in patients with non-alcoholic steatohepatitis (p = 0.005) and cirrhosis (p < 0.001). ^{13}C-caffeine breath test significantly correlated with Brunt's fibrosis histologic score (r = -0.49, p < 0.001) but not with steatosis or inflammation [62].

The validation and usefulness of functional liver tests in the NASH space continues to evolve. These tests are sometimes employed in current NASH drug development trials as secondary or exploratory outcomes.

Potential tests and areas of growth

The need for a reliable, safe and well validated non-invasive liver function test to monitor fibrosis in NAFLD has been recognized by clinicians and experts in industry as well as regulatory authorities. There is a growing need to clinically characterize the stages of NALFD/NASH disease in order to stratify risk of progression to end stage liver disease (ESLD). Given there are more than 30 different clinical trials at various stages of development for NAFLD and because most of the trials uses liver biopsy as the primary study outcome, the availability of a validated, regulatory accepted non-invasive biomarker for NASH would greatly improve the development process in terms of cost containment and inherent patient risk associated with the performance of liver biopsy. These non-invasive biomarkers will need to be cost-effective if they are to be used for patient screening. As we move to commercialized medications for the treatment of NASH payors will want to make sure that a patient has NASH, and, to monitor if the patient is responding to the prescribed medication. These parameters simply cannot be fulfilled by liver biopsy due to the expense, patient risk, availability and variability in interpretation amongst pathologists.

Conclusion

Nonalcoholic fatty liver disease and NASH are disease areas that are growing in prevalence due to a combination of increased clinician recognition and increasing development of associated co-morbidities amongst patients, including type-2 diabetes mellitus and obesity. Currently, the only acceptable methodology for NASH diagnosis and disease progression monitoring is liver biopsy.

There is a plethora of scoring systems, serum markers and radiologic imaging being developed to bridge the gap in providing a non-invasive modality for the diagnosis of NASH. We do know that the progression of NASH and its associated liver-related complications are associated with the degree of fibrosis occurring within the liver. Non-invasive biomarkers are also in development for tracking the stage of liver fibrosis over time; this would provide a modality for assessing the impact of a given treatment on NASH followed over time. These biomarkers continue to evolve with regards to sensitivity, specificity and applicability to a broad patient population. These fibrosis biomarkers can be complemented by testing that assess liver function. These liver function tests can assist in determining when a patient with compensated cirrhosis is developing liver dysfunction and crossing 'the bridge" to a state of decompensated cirrhosis. The development of decompensated cirrhosis is the current definition of liver failure; an important outcome to track when the pharmacologic treatment options for NASH are commercially available.

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