Non-invasive Diagnosis of Fibrosis in Non-alcoholic Fatty Liver Disease

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Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in developed as well as in developing countries. Its prevalence continues to rise currently affecting approximately 20–30% of adults and 10% of children in the United States. Non-alcoholic fatty liver disease represents a wide spectrum of conditions ranging from fatty liver, which in general follows a benign non-progressive clinical course, to non-alcoholic steatohepatitis (NASH), a more serious form of NAFLD that may progress to cirrhosis and end-stage liver disease. Liver biopsy remains the gold standard for evaluating the degree of hepatic necroinflammation and fibrosis; however, several non-invasive investigations, such as serum biomarkers, have been developed to establish the diagnosis and also to evaluate treatment response. There has been a substantial development of non-invasive risk scores, biomarker panels, and radiological modalities to identify at risk patients with NAFLD without recourse to liver biopsy on a routine basis. Examples include combination of serum markers like NAFLD fibrosis score (NFS), BARD score, fibrometer, FIB4, and non-invasive tools like fibroscan which assess fibrosis in patients with NAFLD. Other markers of fibrosis that have been evaluated include high-sensitivity C-reactive protein, plasma pentraxin 3, interleukin-6, and cytokeratin-18. This review focuses on the methods currently available in daily clinical practice in hepatology and touches briefly on the potential future markers under investigation. (J CLIN EXP HEPATOL 2012;2:145–155)

Non-alcoholic fatty liver disease (NAFLD) encompasses a wide spectrum of conditions associated with over accumulation of fat in the liver which range from benign fatty liver to an intermediate stage of non-alcoholic steatohepatitis (NASH) leading to advanced fibrosis and cirrhosis. Together, NASH and hepatic steatosis collectively affect between 20% and 30% of people in developing and developed countries, respectively, making NAFLD the commonest liver ailment in the world.¹ Non-alcoholic steatohepatitis, the more advanced and clinically important form of NAFLD, is less common, with an

estimated prevalence of 2–3% in the general population and 16% and 37% in the morbidly obese individuals.^{2,3} Another issue of concern is that NAFLD now affects 3% of the general pediatric population, rising to 53% in obese children^{4,5} with considerable implications for future liver disease burden. The most common benign form of NAFLD is fatty liver and represents about 80–90% of cases, whereas NASH is a potentially serious condition associated with a significant increase in the overall and liver-related morbidity and mortality.^{6,7}

NATURAL HISTORY OF NON-ALCOHOLIC FATTY LIVER DISEASE

The short-term prognosis of NAFLD is largely excellent, but long-term prognosis depends crucially on histological stage at presentation.^{8,9} In patients with bland steatosis, two studies have reported either nil¹⁰ or minimal progression¹¹ to advanced disease over a median of 11.5 and 16.7 years, respectively. For those with NASH on baseline liver biopsy, 11% went on to develop cirrhosis and 40% of patients die from any cause within 15 years of which 7% are from liver-related complications, especially in those with advanced fibrosis or cirrhosis. Studies with liver biopsy on follow-up have also prospectively evaluated the risk of fibrosis progression over time.^{12,13} One hundred and three patients underwent two liver biopsies 1–21 years apart: Baseline low fibrosis stage, diabetes, and greater body mass index (BMI) were independently associated with fibrosis progression.¹⁴ In another study, 70 patients underwent two liver biopsies 10–16 years apart; progression of

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Abbreviations: ALP: alkaline phosphatase; ALT: alanine aminotransferase; APRI: AST to platelet ratio index; AST: aspartate aminotransferase; AUCs: area under the curves; AUROC: area under the receiver-operating characteristics curve; BMI: body mass index; CCL2: CC-chemokine ligand-2; CI: confidence interval; CT: computed tomography; ECM: extracellular matrix; ELF: European liver fibrosis; FT: FibroTest; GGT: gamma-glutamyl-transpeptidase; HA: hyaluronic acid; HTGC: hepatic triglyceride levels; LSM: liver stiffness; MRE: magnetic resonance elastography; MRI: magnetic resonance imaging; MRS: magnetic resonance spectroscopy; NAFL: non-alcoholic fatty liver; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; NFS: NAFLD fibrosis score; NPV: negative-predictive value; OR: odds ratio; PPV: positive-predictive value; SF: serum ferritin; SPCA: Serum proline dipeptidase activity; TE: transient elastography; TIMP1: tissue inhibitor of metalloproteinase 1; TNF: tumor necrosis factor; TRX: thioredoxin; US: ultrasonography
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fibrosis stage occurred in 41% and was associated with diabetes, weight gain, and increased insulin resistance.¹⁵ An important issue in patients with NAFLD is to differentiate NASH from simple steatosis as NASH patients are at sustained risk of developing complications of chronic liver disease (CLD), such as cirrhosis, liver failure, and hepatocellular carcinoma.

The diagnosis of NASH, which is characterized by necro-inflammation, ballooning degeneration, and fibrosis, is essentially based on histological examination by liver biopsy. The liver biopsy removes only about 1/50,000th of the liver which is a painful and invasive procedure with potentially life-threatening complications.^{16,17} Complicating the ability of a liver biopsy to diagnose NASH is inter-observer and intra-observer variabilities between histopathologists. Grading of steatosis is reliable with Kappa statistics for intra-observer and inter-observer variabilities between 0.74–0.98 and 0.79, respectively. However, fibrosis staging, lobular inflammation, and ballooning interpretation have relatively high intra-observer and inter-observer variabilities.^{18–21} In addition, given the numbers of patients with NAFLD, the use of liver biopsy is clinically and financially impractical. These limitations have stimulated the search for new non-invasive approaches for diagnosis of fibrosis in liver. Ideally, a non-invasive marker of liver fibrosis should be liver-specific, easy to perform, reliable, reproducible, and inexpensive. It should, in addition, be accurate not only for the staging of fibrosis, but also for monitoring disease progression. For all of these very reasons, there is an urgent need to develop and validate simple, reproducible, non-invasive tests that accurately distinguish NASH from non-alcoholic fatty liver (NAFL) or simple steatosis and determine if possible both the stage and grade of the disease.

LABORATORY TESTS

Most patients with NAFLD, including adults and children with either NAFL or NASH, are asymptomatic at presentation. The features more consistently found to be associated with disease severity include obesity, older age, diabetes, and hypertension. Laboratory tests that are routinely included in the evaluation of patients with suspected NAFLD include a serum panel of liver tests (alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase [ALP], gamma-glutamyl-transpeptidase [GGT], and albumin), prothrombin time, and complete blood count.

Elevated serum ALT and AST levels are the primary abnormality seen in patients with NAFLD and tend to be higher in patients with NASH as compared with NAFL. However, ALT levels are seldom higher than five times the upper limit of normal, and typically fluctuate with normal levels seen in more than two-thirds of patients with NASH over a period of time. Mofrad et al²² demonstrated

that the one can find the entire histological spectrum of NAFLD with normal ALT values. In a similar study by Fracanzani et al²³ 455 patients with NAFLD were divided in two groups according to their serum ALT levels. They compared clinical and histological features of patients with and without increased serum ALT. Non-alcoholic steatohepatitis was diagnosed in 62% and 74% of patients with normal or increased ALT levels, respectively. There were no significant differences in advanced fibrosis between the two groups with or without raised serum ALT, underlying the need for liver biopsy for a definitive diagnosis and staging of fibrosis in NASH.

Among other routine laboratory tests, a reversal of the AST/ALT ratio (AAR) to >1 had been consistently reported to predict the presence of more advanced fibrosis. This observation is also true in a variety of other CLDs.²⁴ In addition to its utility as an individual marker, the AAR is also a component of several other fibrosis scoring systems including the NFS and BARD score (BMI, AAR, and the presence of type 2 diabetes mellitus).²⁵ Despite its simplicity, using a cut-off of 0.8 McPherson et al²⁶ demonstrated a sensitivity (74%), specificity (78%), and negative-predictive value (NPV) of 93% for the diagnosis of advanced fibrosis in NAFLD using the AAR.

Increased serum GGT level has also been shown to be associated with advanced fibrosis in NAFLD, with a study of 50 patients with NAFLD demonstrating an area under the receiver-operating characteristics curve (AUROC) of 0.74 for the prediction of advanced fibrosis. Using a cut-off serum GGT value of 96.5 U/L, GGT predicted advanced fibrosis with 83% sensitivity and 69% specificity.²⁷

The AST to platelet ratio index (APRI) was originally developed for use in chronic hepatitis C. In a recent meta-analysis, the summary area under the curves (AUCs) of the APRI for significant fibrosis and cirrhosis were 0.76 (95% confidence interval [CI], 0.74–0.79) and 0.82 (95% CI, 0.79–0.86), respectively, for hepatitis C related fibrosis. For significant fibrosis, an APRI threshold of 0.5 was 81% sensitive and 50% specific.²⁸ Its utility in NAFLD has since been studied by a number of researchers. Using this score, Cales et al²⁹ demonstrated an AUROC of 0.866 for significant fibrosis, 0.861 for severe fibrosis, and 0.842 for cirrhosis in a study of 235 NAFLD subjects. However, significantly lower values were obtained in other studies, where AUROCs of 0.564 for significant fibrosis, 0.568 for advanced fibrosis, and 0.786 for predicting cirrhosis were demonstrated.^{30–32}

Serum ferritin (SF) levels are commonly elevated in patients with NAFLD because of systemic inflammation, increased iron stores, or both. Manousou et al³³ studies 111 patients and found on multivariate regression that diabetes, SF concentrations, BMI, and AST were independently associated with NASH. In a recent study by Kowdley et al³⁴ who studied SF in 628 adult patients with NAFLD

(age, ≥ 18 years) with biopsy-proven NAFLD and an SF measurement within 6 months of their liver biopsy, it was concluded that $SF > 1.5 \times$ upper limit of normal (ULN) is associated with hepatic iron deposition, a diagnosis of NASH, and worsened histologic activity and is an independent predictor of advanced hepatic fibrosis among patients with NAFLD. Furthermore, elevated SF is independently associated with higher NAS, even among patients without hepatic iron deposition.

Laboratory signs of advanced liver disease, such as hyperbilirubinemia, hypoalbuminemia, and abnormal prothrombin time, are seen only in cases associated with cirrhosis.

IMAGING TECHNIQUES

Non-invasive techniques such as ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and proton magnetic resonance spectroscopy (1H-MRS) can detect hepatic steatosis, but cannot reliably distinguish simple steatosis from NASH.

ULTRASOUND

Ultrasonography (US) is currently the preferred method for screening asymptomatic patients with elevated liver enzymes and suspected NAFLD. Ultrasonography findings of fatty liver include hepatomegaly, diffuse increase in echogenicity of the liver parenchyma, and vascular blunting. The sensitivity of US in detecting steatosis varies between 60% and 94%,³⁵ and also varies depending on steatosis degree. Sensitivity is very low when the degree of steatosis is $<30\%$; this technique has important limitations. First, it is operator-dependent and subject to significant intra- and inter-observer variability,³⁶⁻³⁸ second, US does not provide quantitative information of the degree of lipid accumulation. Another limitation is its inability to differentiate simple steatosis from steatohepatitis as both of them have similar appearance on ultrasound.

DIFFERENTIATION OF NON-ALCOHOLIC FATTY LIVER DISEASE FROM NON-ALCOHOLIC STEATOHEPATITIS

A simple non-invasive and easy to perform parameter is longitudinal diameter of the spleen. As the study of Tarantino et al³⁹ recently showed, spleen diameter could differentiate between NAFLD and NASH better than both IL-6 and vascular endothelial growth factor, with values >116 mm predicting NASH. Iijima et al⁴⁰ evaluated the utility of contrast ultrasound with levovist for the diagnosis of NASH. Levovist, an ultrasound contrast agent, was injected intravenously and the liver was scanned at different time intervals. They found a statistically significant decrease of signal intensity in NASH, when compared with NAFLD, due to reduced uptake of levovist mediated by cell injury. However, larger studies are needed to establish the role of contrast US in the diagnosis of NASH.

COMPUTED TOMOGRAPHY

Non-contrast-enhanced CT is the most accurate CT technique to detect and characterize hepatic steatosis.⁴¹ The CT diagnosis of hepatic steatosis is made by measuring the difference in liver and spleen attenuation values in Hounsfield units. The density of the liver, as visualized by CT, decreases as the severity of steatosis increases. Computed tomography can also visualize splenomegaly as a sign of portal hypertension, which is suggestive for advanced fibrosis in patients with NAFLD. In a study by Park et al⁴² 154 candidates, including 104 men and 50 women, underwent unenhanced CT and US-guided liver biopsy on the same day. They concluded that diagnostic performance of unenhanced CT for quantitative assessment of macrovesicular steatosis is not clinically acceptable. Unenhanced CT, however, provides high performance in qualitative diagnosis of macrovesicular steatosis of 30% or greater. However, CT exposes subjects to ionizing radiation and cannot assess concurrent inflammation and fibrosis.

MAGNETIC RESONANCE IMAGING AND PROTON MAGNETIC RESONANCE SPECTROSCOPY

Magnetic resonance imaging provides an accurate and rapid assessment of hepatic steatosis to a lower limit of 3%. Phase-contrast imaging correlates with the quantitative assessment of fatty infiltration across the entire range of liver diseases. A new MRI technique, proton MRS, measures the fat proton fraction and hepatic triglyceride levels (HTGC). The 95th percentile of HTGC in subjects (Dallas Heart Study) was 5.56%, which corresponds to a HTGC of 55.6 mg/g. Thus, MRS provides a sensitive, quantitative, non-invasive method to measure HTGC and, when applied to a large urban US population, revealed a strikingly high prevalence of hepatic steatosis.⁴³ Reproducibility of (1) H-MRS to measure HTGC for 'between-weeks' measurements and in fatty livers is high, which is important for follow-up studies.⁴⁴ Magnetic resonance spectroscopy characterizes metabolic processes involved in cellular regeneration, and thus it can evaluate the disease severity in NASH. An increased ATP/phosphate ratio might be a signal for progression to an advanced stage of fibrosis in NASH. Magnetic resonance spectroscopy is probably more accurate than previous imaging procedures for the diagnosis of NAFLD but it needs validation in large human studies. In a study by Guiu B et al,⁴⁵ patients underwent 3-T single-voxel point-resolved (1) H MR spectroscopy of the liver (Couinaud segment VII) which was performed to calculate the liver fat fraction from the water (4.7 ppm) and methylene (1.3 ppm) peaks, corrected for T1 and T2 decays. Liver fat fraction was also computed from triple-echo (consecutive in-phase, opposed-phase, and in-phase echo times) breath-hold spoiled gradient-echo sequence (flip angle, 20°), by estimating T2* and relative signal

intensity loss between in- and opposed-phase values, corrected for $T2^*$ decay. They concluded that a breath-hold triple-echo gradient-echo sequence with a low flip angle and correction for $T2^*$ decay is accurate for quantifying fat in segment VII of the liver. Given its excellent correlation and concordance with (1)H MR spectroscopy, this triple-echo sequence could replace (1)H MR spectroscopy in longitudinal studies if and when validated in large number of patients with NAFLD.^{45,46}

Both computerized tomographic scanning and, in particular, MRI seem to be more sensitive techniques for quantification of steatosis. However, none of these imaging techniques have sufficient sensitivity and specificity for staging the disease and cannot distinguish between simple steatosis and NASH with or without fibrosis.

MAGNETIC RESONANCE ELASTOGRAPHY

An MR equivalent of transient elastography (TE) has recently demonstrated excellent diagnostic accuracy with sensitivity and specificity of 98% and 99%, respectively, for detecting all grades of fibrosis.⁴⁰ In a prospective study by Huwart et al,⁴⁷ prospective blind comparison of magnetic resonance elastography (MRE), ultrasound elastography, and APRI in a 141 consecutive series of patients who underwent liver biopsy for CLD was studied. The areas under the receiver-operating characteristic curves of MRE (0.994 for $F \geq 2$; 0.985 for $F \geq 3$; 0.998 for $F = 4$) were larger ($P < 0.05$) than those of ultrasound elasticity, APRI, and the combination of ultrasound elasticity and APRI. Magnetic resonance elastography was also associated with a higher technical success rate than US elastography and hepatic stiffness did not appear to be affected by steatosis using this technique which had been a previous concern. However, this technique remains experimental at this time.

FIBROSCAN

Transient elastography (Fibroscan, Echosens, Paris, France) is a non-invasive method of assessing liver fibrosis which can be performed at the bedside or in the outpatient clinic. It employs ultrasound-based technology to measure liver stiffness (LSM), and has been validated for use mainly in patients with chronic hepatitis C and chronic hepatitis B.^{48,49} However, recent studies had shown good results in patients with NAFLD.^{50,51} Failure to obtain a reading occurs in only 5% of cases, but is more common in obese patients which has so far limited its use in the NAFLD cohort, although a recently introduced XL probe may reduce this problem.⁵² Although fibroscan is less well validated in NAFLD, liver stiffness measurements in 67 NAFLD patients (mean [SD] age, 50.4 [3.3] years) in whom the diagnosis had been confirmed by liver biopsy and the severity of fibrosis had been scored according to Brunt. The median liver stiffness values (with 95% CIs) were F0, 4.907 (4.417–5.396) kPa; F1, 6.142 (5.582–6.702) kPa; F2,

7.894 (6.384–9.404) kPa; F3, 11.027 (8.555–13.500) kPa; F4, 26.960 (17.705–36.215) kPa. Stepwise increase in liver stiffness with increasing histological fibrosis was demonstrated in this study of NAFLD patients.⁵³ In a recent study, 246 consecutive patients from two ethnic groups had successful liver stiffness measurement and satisfactory liver biopsy specimens. The AUROC of TE for F3 or higher and F4 disease was 0.93 and 0.95, respectively, and was significantly higher than that of the AAR, APRI, FIB-4, BARD, and NFSs (AUROC ranged from 0.62 to 0.81, $P < 0.05$ for all comparisons). At a cut-off value of 7.9 kPa, the sensitivity, specificity, and positive- and NPVs for F3 or greater disease were 91%, 75%, 52%, and 97%, respectively. With high NPV and modest positive-predictive value (PPV), TE is useful as a screening test to exclude advanced fibrosis. Liver biopsy may be considered in NAFLD patients with liver stiffness of at least 7.9 kPa.⁵¹ In a recent meta-analyses for NASH with advanced fibrosis, pooled AUROC, sensitivity and specificity of NFS, and fibroscan are 0.85 (0.80–0.93), 0.90 (0.82–0.99), 0.97 (0.94–0.99) and 0.94 (0.90–0.99), 0.94 (0.88–0.99) and 0.95 (0.89–0.99), respectively.⁵⁴ These values are different from patients with HCV-related fibrosis as in a study by Castéra et al⁵⁵ where fibroscan values ranged from 2.4 kPa to 75.4 kPa (median, 7.4 kPa). Cut-off values were 7.1 kPa for $F \geq 2$, 9.5 kPa for $F \geq 3$, and 12.5 kPa for $F = 4$. Although further studies will undoubtedly add to the current evidence base, fibroscan has now been validated in NAFLD, and represents a useful tool for rapid, non-invasive assessment of liver fibrosis and determining the need for biopsy. Fibroscan evaluates liver stiffness, which is related to fibrosis, inflammation, and portal hypertension. Therefore, fibroscan values have to be interpreted according to clinical, biological, and morphological data.

Markers of Fibrosis

As individual clinical or biochemical markers are not sufficiently accurate to predict the presence of NASH, multiple parameters have been combined into mathematical models to produce diagnostic algorithms to facilitate the non-invasive assessment of NAFLD and differentiation between different stages of NAFLD. Such scoring systems have generally demonstrated greater utility in the detection of advanced fibrosis than intermediate and early stages of fibrosis, a group which potentially is more likely to benefit from therapeutic interventions.⁵⁶

The BARD score is a simple scoring system designed to identify NAFLD patients with a low-risk of advanced disease. It combines three variables of BMI, AAR, and the presence of diabetes into a weighted sum (BMI $> 28 = 1$ point, AAR of $> 0.8 = 2$ points, DM = 1 point), to generate a score from 0 to 4. In the original study, a score of 2–4 was shown to be associated with an odds ratio (OR) for advanced fibrosis of 17 and a NPV of 96%.⁴⁹ The BARD score was also validated in a Polish NAFLD cohort, where

an NPV of 97% was demonstrated, but appeared less useful in a Japanese cohort, where the AUROC was 0.73 with NPV 77%.^{57,58}

The FIB-4 test combines age with three standard biochemical values (platelets, ALT, and AST) to assess fibrosis. In NAFLD, FIB-4 has demonstrated similar results to the AAR where, using a cut-off of 1.3, its sensitivity 85%, specificity 65%, and NPV of 95% were demonstrated for the diagnosis of advanced fibrosis. In another study in 541 patients with NAFLD, FIB-4 had the highest PPV and NPV of 80% and 90%, respectively, for the diagnosis of advanced fibrosis.^{58–60} In a study by Sumida et al⁶¹ in Japanese cohort of 576 patients with biopsy-proven NAFLD, FIB4 index was superior to those for the other scoring systems for differentiating between advanced and mild fibrosis.

FibroMeter is a panel of serum markers which was originally developed for staging fibrosis in chronic HCV.^{62,63} However, fibrometer NAFLD has since been developed which has shown good diagnostic accuracy in staging NASH-related fibrosis. This panel combines seven variables (age, weight, fasting glucose, AST, ALT, ferritin, and platelet count), and in a study of 235 NAFLD patients the highest accuracy 91% for significant fibrosis was obtained with the fibrometer whose (i) AUROC (0.943) was significantly higher than those of NAFLD fibrosis score (NFSa, 0.884, $P=0.008$) and APRI (0.866, $P<0.0001$ vs NFSa) in the whole population.²⁹

The NFS is a panel comprising six variables of age, hyperglycemia, BMI, platelet count, albumin, and AAR, which was constructed using a large panel of 733 biopsy-proven NAFLD. In a recent meta-analysis, NFS achieved pooled sensitivity and specificity of 0.90 (0.82–0.99) and 0.97 (0.94–0.99) for the identification of NASH with advanced fibrosis.⁵⁴ The NFS thus facilitates the identification of NAFLD patients with more advanced disease who require ongoing follow-up, and considerably reduces the requirement for liver biopsy in the minority of patients with an indeterminate score. In a recent study by Armstrong et al⁶⁴ in a primary-care cohort data from 1118 adult patients were analyzed. The cause of abnormal liver functional tests was identified in 55% (614/1118) of subjects, with NAFLD (26.4%; 295/1118) and alcohol excess (25.3%; 282/1118) accounting for the majority. A high NFS (>0.676) suggesting the presence of advanced liver fibrosis was found in 7.6% of NAFLD subjects, whereas 57.2% of NAFLD patients had a low NFS (<-1.455) allowing advanced fibrosis to be confidently excluded. Of these various algorithms, FIB-4 and the NFS have been validated most widely and have demonstrated superior test characteristics.

Liver fibrosis represents the most worrisome histopathological feature in patients with NAFLD, because it suggests a more severe and progressive liver damage. Thus, as in other liver diseases, accurate assessment of the extent of fibrosis is essential for patients with NAFLD. Several

groups have developed various markers using a panel of markers of clinical and biochemical tests to generate clinical models of fibrosis, whereas others focused on specific markers of fibrosis either in isolation or in multicomponent tests.

Ratziu et al⁶⁵ combined four clinical variables to generate the BAAT score: BMI (≥ 28 Kg/m²), age (≥ 50 years), ALT (≥ 2 normal), and serum triglycerides (≥ 1.7 mmol/L). The presence of each variable gives 1 point in the combined score. Whereas a total score of 0 or 1 had a NPV of 100% for fibrosis, a high total score of 4 gave a sensitivity of 14% and a specificity of 100% for the detection of septal fibrosis.

FibroTest (FT) is another validated marker for the quantitative assessment of fibrosis in NAFLD, alcoholic liver disease (ALD), and chronic viral hepatitis. In a meta-analysis by Shaheen et al,⁶⁶ 12 studies were identified, 9 for FT ($n=1679$), and 4 for fibroscan ($n=546$). In heterogeneous analyses for significant fibrosis, the AUCs for FT and fibroscan were 0.81 (95% CI 0.78–84) and 0.83 (0.03–1.00), respectively. At a threshold of approximately 0.60, the sensitivity and specificity of the FT were 47% (35–59%) and 90% (87–92%), respectively. Combining five biochemical markers of haptoglobin, a2-macroglobulin, apolipoprotein A1, total bilirubin, and GGT, corrected for age and gender, a mean standardized AUROC of 0.84 for advanced fibrosis in patients with NAFLD was demonstrated using FT in one meta-analysis in which a total of 30 studies were included which pooled 6378 subjects with both FT and biopsy (3501 HCV, 1457 HBV, 267 NAFLD, 429 ALD, and 724 mixed). The AUROC for the diagnosis of the intermediate adjacent stages F2 vs F1 (0.66; 0.63–0.68, $n=2055$) did not differ from that of the extreme stages F3 vs F4 (0.69; 0.65–0.72, $n=817$) or F1 vs F0 (0.62; 0.59–0.65, $n=1788$).⁶⁷ The most frequent causes of FT failure include Gilbert's syndrome, cholestasis, and acute inflammation, which result in increases in bilirubin and haptoglobulin, respectively, and an abnormal apolipoprotein A1 concentration.

FibroMAX

Among the non-invasive alternatives to liver biopsy, several studies have demonstrated the predictive value and a better benefit-to-risk ratio than liver biopsy of five combinations of simple serum biochemical markers (the super combination being FibroMAX (BioPredictive, Paris, France) in patients at risk of CLDs: FT (BioPredictive) for the quantitative assessment of fibrosis; SteatoTest (BioPredictive) for the quantitative assessment of steatosis; ActiTest (BioPredictive) for the quantitative assessment of necroinflammatory activity in chronic viral hepatitis C and B; NashTest (BioPredictive) for the categorical diagnosis of NASH; and AshTest for the quantitative assessment of alcoholic steatohepatitis (also known in the USA as HCV-FibroSURE, HBV-FibroSURE, ASH-FibroSURE, and NASH-FibroSURE; LabCorp, NC, USA).^{68,69}

Predictive Model for the Diagnosis of Non-alcoholic Steatohepatitis

As individual clinical or biochemical markers are not sufficiently accurate to predict the presence of NASH, multiple parameters have been combined into mathematical models to produce various diagnostic algorithms (Table 1). Dixon et al⁷⁰ first evaluated liver biopsies taken at laparoscopic obesity surgery in 105 consecutive patients. Twenty-six patients (25%) were found to have NASH, and 11 (42%) of these had advanced fibrosis. A raised index of insulin resistance (OR 9.3, 95% CI 3.4–26), systemic hypertension (OR 5.2, 95% CI 2–13.5), and raised ALA (OR 8.6, 95% CI 3.1–23.5) was the independent predictor of NASH. A combination of two or three of these predictors allows a sensitivity of 0.8 and a specificity of 0.89 for NASH. Palekar et al⁷¹ generated a clinical model to distinguish NASH from simple steatosis by combining six different variables including age (>50 years), female gender, AST (>45 U/L), BMI (>30 Kg/m²), and AAR (>1), and serum hyaluronic acid (HA) (>55 mg/L). The AUC for this model was 0.76. The presence of three or more of these factors had a sensitivity and specificity for NASH diagnosis of 74% and 66%, respectively. Gholam et al⁷² proposed a simplified model created using logistic regression analysis using only AST and diagnosis of diabetes, which was able to separate NASH from fatty liver with or without non-specific inflammation in bariatric surgery patients with similar accuracy as the panels described in previous studies.

Poynard et al⁷³ developed a model (the NASHTest) using the combination of 13 variables including age, gender, height, weight, and serum levels of triglycerides, cholesterol, alfa2macroglobulin, apolipoprotein A1, haptoglobin, GGT, ALT, AST, and total bilirubin. One hundred and sixty patients with NAFLD were prospectively included in a training group, 97 were included in a multicenter validation group, and 383 controls. For the diagnosis of definite NASH defined by a NAFLD activity score of greater than 4, both sets had an AUC of 0.79. For the diagnosis of NASH as determined by the grading pathologist, the NASH test provided AUC in the training and validation sets of 0.78 and 0.69, respectively.

Fibrosis is a dynamic process which may result in increased circulating levels of extracellular matrix (ECM) components. Several groups have used this reasoning to develop different blood tests using individual or a composite of ECM components. Suzuki et al⁷⁴ determined the reliability of serum HA to predict the severity of hepatic fibrosis in 79 patients with histologically confirmed NAFLD. Serum HA was obtained at the time of liver biopsy. The logarithm of serum HA showed a significant positive correlation with the stage of fibrosis, and this association persisted after adjusting for age and serum albumin. The test was found to be useful for predicting severe fibrosis (stages 3–4) with AUC of 0.9 (95% CI: 0.83, 0.97). The study could not evaluate the efficacy for moderate fibrosis (stage 2) due to the limited number of patients

Table 1 Serum markers of fibrosis in non-alcoholic fatty liver disease.

Index	No. of patients	Factors	Fibrosis stage	Cut-off	PPV (%)	NPV (%)	AUC
BAAT ⁶⁵	93	Age, BMI, serum, ALT, triglycerides	F $\geq$ 2 vs $\leq$ 1	2	61	86	0.84
OELF ⁷⁶	61	Age, HA, TIMP-1, PIIINP	F $\geq$ 3 vs $\leq$ 2	0.375 0.462	80 87	98 96	0.87
ELF plus simple Clinical marker ⁷⁷	192	HA, TIMP-1, PIIINP plus NFS	F $\geq$ 1 F $\geq$ 2 F $\geq$ 3	-5.002 -0.995 -0.2826	86 94 77	66 75 99	0.84 0.93 0.98
HA ⁸²	79	HA	F $\geq$ 3 vs $\leq$ 2	46.1	51	96	0.92
NFS ⁵⁴	480 253	Hyperglycemia, BMI, platelet count, albumin, AST:ALT, age	F $\geq$ 3 vs $\leq$ 2	<-1.455 >0.676	56 90	93 85	0.88 0.82
NS ⁸⁶	112	Type 4 collagen, HA	F $\geq$ 3 vs $\leq$ 2	COLL $\geq$ 5 or HA $\geq$ 50	66	95	NS
FT ⁶⁹	170 97	Total bilirubin, GGT, gender, age, α 2-macroglobulin, apolipoprotein, A1, haptoglobin	F $\geq$ 2 vs $\leq$ 1 F $\geq$ 3 vs $\leq$ 2	0.30 0.70 0.30 0.70	54 98 71 97	90 76 98 89	0.81 0.88
BARD ⁵⁶	827 160	Diabetes mellitus, BMI, ALT:AST	F $\geq$ 3	2–4	43	96	0.81

ALT: alanine aminotransferase; AST: aspartate aminotransferase; AUC: area under the curve; BMI: body mass index; ELF: enhanced liver fibrosis; FT: FibroTest; GGT: gamma-glutamyl transpeptidase; HA: hyaluronan; NFS: NAFLD fibrosis score; NPV: negative-predictive value; NS: not significant; OELF: original European liver fibrosis; PIIINP: N-terminal propeptide of type III pro-collagen; PPV: positive-predictive value; TIMP: tissue inhibitors of metalloproteinases.

with this stage of fibrosis and showed low accuracy for mild fibrosis.

The original European liver fibrosis (ELF) test is a panel of automated immunoassays to detect three markers of matrix turnover in serum: HA, tissue inhibitor of metalloproteinase 1 (TIMP1), and aminoterminal peptide of procollagen III (P3NP), used in combination with age.⁷⁵ The simplified ELF panel excludes age but has a similar diagnostic performance. The addition of five simple markers—BMI, presence of diabetes/impaired fasting glucose, AAR, platelets, and albumin—to the ELF test improved diagnostic accuracy further, with AUROCs of 0.98, 0.93, and 0.84 for the diagnosis of severe, moderate, and no fibrosis, respectively.⁷⁶ The ELF panel may also represent a useful prognostic tool, with a 1-unit change in ELF score shown to be associated with a doubling of the odds of significant liver-related mortality or morbidity at a 6-year follow-up.⁷⁷

Biomarkers of Hepatocyte Apoptosis, Inflammation, and Oxidative Stress

Apoptosis plays an important role in liver injury and disease progression in NAFLD. An increase in hepatocyte cell death by apoptosis is typically present in humans with NASH as well as in animal models of NASH but absent in those with simple steatosis. Another interesting approach is represented by the evaluation of plasma caspase-3-generated cytokeratin-18 (CK-18) fragments, a biomarker of hepatocyte apoptosis. Levels of CK-18 fragments were able to identify patients with NASH as compared with those with bland steatosis with remarkably high specificity and acceptable sensitivity. Using alone^{78–80} or combination⁸¹ of markers which included cleaved and intact CK-18 levels, adiponectin, and resistin, a model with AUC values between 0.73 and 0.91, was developed in a cohort of 101 patients. Subsequently, the same panel was re-evaluated in another sample of 79 patients and had a lower AUC of 0.70. This led to the development of another model (the NASH diagnostic panel) consisting of diabetes, gender, BMI, triglyceride, CK-18 fragments, and total CK-18 levels.⁸² This new model had higher AUC levels than NASH diagnostics or CK-18 fragment levels in isolation; however, this model has not been validated in an independent sample.

Other potential fibrosis biomarkers include type VI collagen 7S domain and HA, with the latter also representing a constituent of the ELF test. In a cohort of 112 NAFLD subjects, these two biomarkers were able to exclude advanced fibrosis with AUROCs of 0.82 and 0.80, and NPVs of 84% and 78%, respectively. These biomarkers also demonstrated PPVs of 86% and 92% and AUROCs of 0.83 and 0.80 for discriminating NASH from simple fatty liver.⁸³

Other studies of HA in NAFLD using varying cut-off levels have demonstrated AUROCs of between 0.89 and 0.97 for detecting advanced fibrosis. Kaneda et al⁸⁴

demonstrated HA to have an AUROC, NPV, sensitivity, and specificity of 0.97%, 100%, 100%, and 89%, respectively, for detecting severe fibrosis, with a lower AUROC of 0.87 demonstrated for type IV collagen. Lesmana and Yoneda et al^{85,86} also demonstrated the utility of levels of HA and type IV collagen to differentiate between mild (F1-2) and advanced fibrosis (F3-4), and in a separate study of 80 patients with NAFLD, the combination of HA with AST, AAR, age, gender, and BMI demonstrated an AUROC of 0.763 for distinguishing simple steatosis and NASH.⁷¹ In a small study of serum ECM components in 30 patients with NAFLD, serum laminin >282 ng/mL was shown to have an accuracy of 87%, sensitivity 82%, specificity 89%, PPV 82%, and NPV 89% for identifying the presence of NASH with fibrosis.⁸⁷

A growing body of evidence supports a central role for inflammation and inflammatory cytokines in the development of the metabolic syndrome and NAFLD. A cytokine imbalance, and in particular an increase in the tumor necrosis factor- α (TNF- α)/adiponectin ratio, may play an important role in the development of NASH.⁷⁸ Two other inflammatory markers, interleukin-6 (IL-6) and high-sensitive C-reactive protein (hs-CRP), which have been extensively studied as markers for insulin resistance, type-2 diabetes, and cardiovascular disease, have been tested in patients with NAFLD. Hui et al⁷⁸ quantified serum levels of both TNF- α and adiponectin in patients with biopsy-proven NAFLD. Adiponectin levels were significantly lower and TNF- α levels were significantly higher in patients with NASH as compared with controls. However, when compared with simple steatosis, only adiponectin levels remained significantly lower in NASH patients, whereas no difference was noted in TNF- α levels between the two groups of patients. In another study, hs-CRP, IL-6, and several chemokines, including, were measured in 47 patients with biopsy-proven NAFLD as well as 30 age-matched, sex-matched, and ethnicity-matched controls. Both IL-6 and CCL2 but not hs-CRP were increased in patients with NAFLD as compared with controls. Moreover, only CCL2 levels were different between patients with NASH and those with simple steatosis.⁸⁸ In a large cohort of patients ($n=120$) in a study by Hui et al, there were significant associations between the hs-CRP levels and age ($r=0.2$, $P=0.01$), BMI ($r=0.5$, $P=0.01$), waist-to-hip ratio ($r=0.4$, $P=0.001$), and insulin resistance by homeostasis model ($r=0.2$, $P=0.04$). However, no relationship was discerned between the levels of hs-CRP and the grades of hepatic steatosis ($r=0.2$, $P=0.1$), necroinflammation ($r=0.1$, $P=0.4$), and fibrosis ($r=0.1$, $P=0.5$). The mean ($\pm$ SD) hs-CRP for NASH was 2.68 ± 2.10 mg/L, and 2.23 ± 1.68 mg/L for simple steatosis ($P=0.4$).⁸⁹

Markers of stress and inflammation like IL-6 and thioredoxin (TRX) which is stress-inducible thiol-containing protein may represent a clinically useful indicator of oxidative stress and found to be increased in patients with

Table 2 Plasma candidate biomarkers for distinguishing simple steatosis from non-alcoholic steatohepatitis.

Author	Markers	Functions	Results	Area under curve
Hui et al ⁷⁸	Adiponectin	Adipose tissue metabolism/adipokines	Reduced in NAFLD compared with controls, lowest levels in NASH	0.79
Lemoine et al ⁷⁹	Leptin	Adipose tissue metabolism/adipokines	HOMA were correlated positively with leptin levels	0.82
Tarantino et al ⁹⁰	IL-6	Inflammation	Increased in patients with NASH	0.817
Sumida et al ⁹¹	Thioredoxin	Stress-inducible protein	Increased in NASH	0.785
Charlton et al ⁹³	Dehydroepiandrosterone	Oxidative stress	Low circulating DHEA-S	0.83
Feldstein et al ⁸⁰	Cytokeratin-18 (CK-18)	Hepatocyte apoptosis	Increased in patients with NASH	0.83
Yilmaz et al ⁹⁹	sRage	Glycation end products adipose tissue metabolism	Reduced in NASH	0.77
Kayadibi et al ⁹²	Serum prolidase enzyme activity	Oxidative stress	Increased in NASH	0.85
Kukla et al ⁹⁹	Chemerin	Adipose tissue metabolism/adipokines	Increased in NASH	–
Alkhoury et al ⁹⁶	Retinol-binding protein 4	Adipose tissue metabolism/adipokines	Lower level in NASH	–
Degertekin et al ⁹⁷	Endothelin 1	Hepatic fibrogenesis	Higher level in NASH	–
Yilmaz et al ⁹⁸	Angiotensin-like protein 3	Plasma triglyceride clearance	Higher level in NASH	–
Tamimi et al ⁹⁴	Soluble Fas	Hepatocyte apoptosis	Higher level in NASH	0.79

DHEA-S: dehydroepiandrosterone; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis.

NASH.^{90,91} Serum prolidase enzyme activity (SPEA) catalyzes the final step of collagen breakdown by liberating free proline for collagen recycling, and is reported to be of hepatic origin which has also been shown to increase in patients with NASH.⁹² Indicators of oxidative stress,^{90,93} apoptosis,^{94,95} and various adipokines^{96–98} have been investigated as surrogate markers of NASH in smaller studies which need more validation. However, most studies to date have been small with mixed results and it is not yet clear whether or not oxidative stress in the liver is accurately reflected in the serum (Table 2).^{99,100}

FUTURE AREAS FOR NON-INVASIVE TESTS

As important progress is made in the elucidation of the pathogenesis of NAFLD, new rational non-invasive serum biomarkers that reflect the pathobiology of the disease, including markers of oxidative stress (OS), inflammation, apoptosis, and fibrosis, are being tested. All these markers are in the initial phases of investigation, and the clinical utility of these tests remains to be determined.¹⁰¹ Recent advances in the field of proteomics have provided us with powerful tools for discovering biomarkers in the field of NAFLD. Among the different technologies available, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) are attractive techniques since they provide protein profiles in an easy and rapid manner from small amounts of protein, obtained from various biologically complex samples. Younossi et al¹⁰⁰ investigated, by

means of SELDI-TOF, serum protein profiles of different subtypes of NAFLD and identified 12 significantly different protein peaks across the study groups. Using a similar SELDI-TOF technique, Trak-Smayra et al¹⁰² have searched for serum markers of steatosis and NASH in obese non-alcoholic patient ($n=80$) candidates for bariatric surgery. Twenty-four obese patients (30%) had non-significant liver lesions, 32 (40%) had significant steatosis, and 24 (30%) had NASH. Comparison of serum protein profiles according to liver lesions identified three peaks (CM10-7558.4, CM10-7924.2, and Q10-7926.9), the intensity of which significantly increased according to the severity of the liver lesions (steatosis and NASH) and returned to normal after bariatric surgery. The future availability of rapid, high-throughput analytical platforms is likely to facilitate molecular phenotyping of different subtypes (simple fatty liver, borderline NASH, definitive NASH) in the spectrum of NAFLD. In addition, innovative methods like proteomics and glycomics will be helpful to establish fibrosis specific serum protein and glycosylation patterns, respectively, for diagnosis and monitoring of fibrogenesis in patients with NAFLD.

CONCLUSION

Currently, the standard procedure for evaluating the degree of necroinflammation and fibrosis, and for quantifying hepatic steatosis remains liver biopsy. However, this is an invasive procedure with unavoidable risks and limitations like sampling error and inter- and intra-observer variations. However, in most cases of NAFLD, the results

of liver biopsy are not relevant to the choice of treatment, which remains that of metabolic syndrome; moreover, it is difficult to repeat the biopsy after treatment and monitoring the disease progress. Hence, there is an impending need for non-invasive strategies to cover the whole spectrum of NAFLD. Non-invasive investigations, such as various biomarkers, fibrosis scoring panels, and imaging techniques, offer considerable promise in their ability to differentiate simple steatosis from significant fibrosis and to stage liver fibrosis in 80–90% of cases. So, in day-to-day clinical practice, a liver biopsy is needed only in 10–20% of patients more for documentation of liver fibrosis rather than making any substantial change in therapeutic intervention. With the advent of proteomics, metabolomics, and better biomarkers studies coupled with promising functional MRS studies, a day may not be far away when liver biopsy may be obsolete at least in distinguishing between simple steatosis and clinically significant fibrosis. Further testing and validation are needed for these non-invasive procedures to refine their role in clinical practice.

CONFLICTS OF INTEREST

All authors have none to declare.

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