NAFLD AND NASH

Noninvasive biomarkers in NAFLD and NASH — current progress and future promise

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Abstract | Nonalcoholic fatty liver disease (NAFLD) affects 25% of the global adult population and is the most common chronic liver disease worldwide. Nonalcoholic steatohepatitis (NASH) is the active form of NAFLD, with hepatic necroinflammation and faster fibrosis progression. With an increasing number of patients developing NASH-related end-stage liver disease and pharmacological treatments on the horizon, there is a pressing need to develop NAFLD and NASH biomarkers for prognostication, selection of patients for treatment and monitoring. This requirement is particularly true as liver biopsy utility is limited by its invasive nature, poor patient acceptability and sampling variability. This article reviews current and potential biomarkers for different features of NAFLD, namely, steatosis, necroinflammation and fibrosis. For each biomarker, we evaluate its accuracy, reproducibility, responsiveness, feasibility and limitations. We cover biochemical, imaging and genetic biomarkers and discuss biomarker discovery in the omics era.

Nonalcoholic fatty liver disease (NAFLD) affects 25% of the global adult population and is the most common chronic liver disease worldwide[1]. On the basis of disease severity, NAFLD is divided into NAFL and nonalcoholic steatohepatitis (NASH). The latter is characterized by histological lobular inflammation and hepatocyte ballooning and is associated with faster fibrosis progression than NAFL[2]. With ongoing liver injury, some patients will progress to cirrhosis and develop various liver-related complications. In the USA, NASH has already become the second or third leading cause of end-stage liver disease and hepatocellular carcinoma (HCC)[3].

Because of the association between NAFLD and metabolic syndrome, lifestyle modification can resolve hepatic steatosis and NASH and improve liver fibrosis (FIG. 1). However, few patients can achieve the target weight reduction, and even fewer can adhere to lifestyle changes in the long term[4,5]. Therefore, some patients with NASH require pharmacological treatment. At present, there is no registered treatment for NASH, but current guidelines have endorsed the use of vitamin E or pioglitazone in selected patients with biopsy-proven NASH[6–8], and four drugs (obeticholic acid, elafibranor, selonsertib and cenicriviroc) have entered phase III development[9]. According to the current requirements of the FDA and the European Medicines Agency, new drugs for NASH can be conditionally registered if they are shown to resolve NASH without worsening of liver fibrosis or if they improve fibrosis without worsening NASH as assessed by paired liver biopsy samples, whereas full registration depends on the drug effect on clinical outcomes (such as progression to cirrhosis, cirrhotic complications and liver-related deaths). Although a drug can be used in the clinic after conditional registration, it can be withdrawn eventually if it fails to show an effect on clinical outcomes.

Thus, liver biopsy is heavily relied upon to assess the severity of NAFLD, select patients for pharmacological treatment, monitor disease progression or treatment response and develop new drugs. However, liver biopsy is an invasive procedure with a small risk of complications such as bleeding[10]. Although repeated liver biopsy might be acceptable in the clinical trial setting, it is unlikely to be widely accepted in the real world. Besides, liver biopsy is not a genuine gold standard. As a biopsy specimen represents only ~1/50,000 of the liver volume, sampling bias and underestimation of disease severity is common. Studies have also consistently demonstrated considerable interobserver variability in the assessment of NASH features such as lobular inflammation and ballooning (discussed later)[11]. There is an urgent need to find better ways to assess patients with NAFLD.

It is, however, important to note that liver biopsy has been the main reference standard for the evaluation of

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Key points

- When assessing a patient with nonalcoholic fatty liver disease (NAFLD), the key histological features of interest include the degree of steatosis, necroinflammation and fibrosis.
- MRI-estimated proton density fat fraction is currently the most accurate test to quantify hepatic steatosis and can be considered the gold standard.
- Magnetic resonance elastography is the most accurate fibrosis test, yet its use is limited by cost and availability.
- Controlled attenuation parameter and liver stiffness measurement by transient elastography also enables simultaneous assessment of hepatic steatosis and fibrosis, albeit with lower accuracy and success rates than MRI-based methods.
- Plasma cytokeratin 18 (CK18) fragment levels are a marker of hepatocyte apoptosis and represent the most extensively evaluated biomarker of steatohepatitis, although the accuracy is modest.
- A number of gene polymorphisms (such as those in *PNPLA3* and *TM6SF2*) have been shown to correlate with NAFLD and its severity, yet their role in patient assessment remains to be established.

biomarkers of NAFLD. As liver biopsy is imperfect, even a perfect biomarker would seem imperfect on the basis of discordance between the two[12]. One possible solution is to compare their correlations with clinical outcomes such as HCC, cirrhotic complications and liver-related death — after all, identifying patients who will develop these complications is the ultimate reason for liver assessments.

In this Review, we discuss noninvasive biomarkers that are currently available or under development for the assessment of NAFLD. The biomarkers are classified according to their role in the diagnosis of NAFLD, NASH and fibrosis and cirrhosis. Other than biochemical markers, we also discuss imaging studies, genetic tests and new information from the omics era. When applicable, we evaluate biomarkers by their accuracy (diagnosing what they intend to diagnose), reproducibility (providing the same results when repeated), responsiveness (changes in biomarkers corresponding to changes in biological processes), feasibility (required instruments, manpower or setting) and limitations. For brevity, the full details of these biomarker characteristics are recorded in the table accompanying each section. Although this article focuses mainly on the diagnosis of NAFLD, NASH and fibrosis and cirrhosis, it is recognized that different biomarkers are needed for monitoring disease progression or regression, predicting or assessing treatment response, prognostication, monitoring treatment safety and evaluating disease susceptibility. Many of these applications, however, require longitudinal outcome studies and data from clinical trials, and should be a research priority.

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Diagnosis of NAFLD

The diagnosis of NAFLD is based on the detection of hepatic steatosis (steatosis in ≥5% of hepatocytes by histology or intrahepatic triglyceride content ≥5.5% by MRI) and exclusion of other liver diseases by history and appropriate investigations[6,8,13]. Diagnosis is the first step to trigger further evaluation of the severity of NAFLD. Although some biomarkers can quantify hepatic steatosis with varying accuracy, the absolute amount of steatosis is not prognostically important[14]. In fact, steatosis can disappear when NAFLD progresses to cirrhosis, and it is currently believed that this 'burnt-out' NASH is the principal aetiology underlying most cases of cryptogenic cirrhosis[15].

Blood biomarkers and panels

There are several indices and scores developed to assess hepatic steatosis (TABLE 1). The fatty liver index (FLI) comprises BMI, waist circumference and serum levels of triglycerides and gamma-glutamyltransferase (GGT)[16]. FLI has moderate accuracy in diagnosing fatty liver as determined by ultrasonography (area under the receiver-operating characteristic curve (AUROC) 0.84). In a population study in Italy, FLI correlated with insulin resistance and predicted all-cause, liver-related and cancer mortality[17]. The hepatic steatosis index (HSI) was derived and validated in a large cohort of >10,000 individuals who underwent health check-ups[18]. HSI includes five components (serum aspartate aminotransferase (AST):alanine aminotransferase (ALT) ratio, BMI, gender and presence of diabetes mellitus) and has a moderate accuracy to detect fatty liver as determined by ultrasonography (AUROC 0.81)[18]. The key limitation of FLI and HSI is the use of a suboptimal reference standard, as diagnosis of fatty liver by ultrasonography could be operator-dependent and insensitive for mild steatosis[19].

A more sensitive and quantitative reference standard to estimate liver fat content — proton magnetic resonance spectroscopy (^{1}H-MRS) — was used to derive the NAFLD liver fat score[20]. This algorithm includes the presence of the metabolic syndrome and type 2 diabetes mellitus, fasting serum insulin concentration, serum AST levels and AST:ALT ratio. NAFLD liver fat score had very good accuracy to diagnose fatty liver (AUROC 0.86–0.87), which is defined as a liver fat content ≥5.56%[20]. The inclusion of serum insulin level, which is not a routine test, in the formula might limit its wider clinical use.

Panels with more specialized parameters are also available to predict hepatic steatosis. SteatoTest (Biopredictive, Paris, France) was constructed using a combination of the six components of FibroTest–ActiTest (Biopredictive; comprising serum levels of total bilirubin, GGT, α_2-macroglobulin (α_2m), haptoglobin, ALT and apolipoprotein AI) plus BMI and

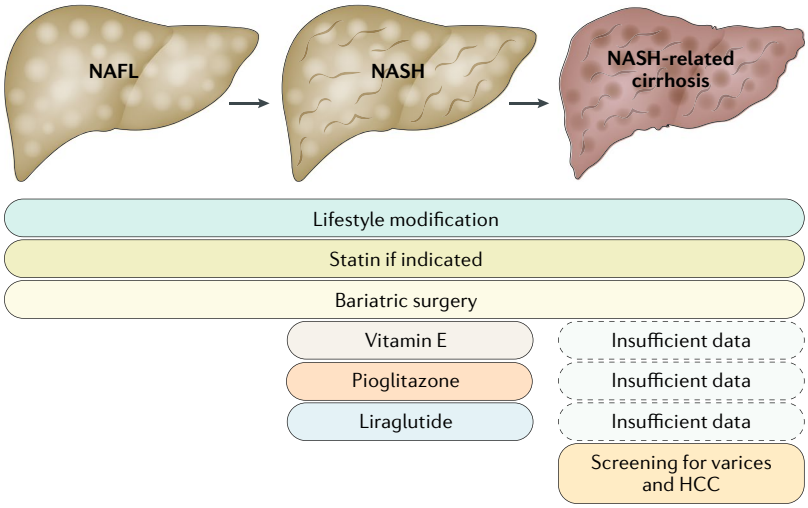

Fig. 1 | **Management of NAFLD according to disease severity.** Resolution of NASH and improvement in fibrosis can be achieved in patients who lose ≥10% body weight, but this target can be achieved in only 10–20% of patients. Although there are no data on the effectiveness of lifestyle modification on outcomes in patients with NASH-related cirrhosis, lifestyle advice would facilitate management of metabolic diseases in these individuals. Statins have a minimal effect on NASH yet are safe to use in patients with chronic liver diseases and have established cardiovascular benefits. Vitamin E can improve NASH but not fibrosis. High-dose vitamin E (≥400 international units daily) might increase all-cause mortality. Pioglitazone can improve NASH but has no or a mild effect on fibrosis. Adverse effects include weight gain, an increased risk of bladder cancer and osteoporosis and potentially fluid retention. Liraglutide promotes weight reduction, can improve NASH and might prevent fibrosis progression. Gastrointestinal adverse effects and the need for injections hinder its use, and efficacy data are limited. Bariatric surgery can reverse NASH and improve fibrosis and is safe in patients with compensated liver disease. However, until further data are available, its use is limited to patients with morbid obesity. Screening for varices and hepatocellular carcinoma (HCC) might reduce the risk of death from these complications. As the absolute incidence of HCC in patients without cirrhosis is very low, screening all patients with non-cirrhotic NAFLD for HCC is not currently cost-effective.

serum levels of total cholesterol, triglycerides and glucose adjusted for age and gender[21]. SteatoTest has moderate accuracy to predict biopsy-proven hepatic steatosis (AUROC 0.79–0.80). Aside from the cost of the biochemical assays, there is an additional charge for calculating the scores using the proprietary Steato Test formula.

In the past decade, the availability of electronic health records has facilitated large-scale epidemiological studies. However, although demographic and laboratory data in such records are robust, clinical observations and anthropometric measurements are often missing. To facilitate registry research, the NAFLD ridge score was developed using a machine learning approach based only on laboratory parameters (serum levels of ALT, HDL cholesterol, triglycerides, haemoglobin A_{1c} (HbA_{1c}) and leukocyte count) and comorbidity data (and the presence of hypertension)[22]. Using ^{1}H-MRS as the reference standard, the NAFLD ridge score has good overall accuracy (AUROC 0.87) and an excellent negative predictive value of 96% to exclude NAFLD. Nonetheless, although these indices can be good for detecting or even quantifying hepatic steatosis in cross-sectional studies, they are less accurate in assessing changes in liver fat over time[23].

Imaging biomarkers

Ultrasonography. Conventional ultrasonography is the most common method for detecting steatosis because of its ready availability and low cost[13]. Using ultrasonography, a steatotic liver appears brighter than surrounding structures because of the increased soundwave scatter and attenuation from lipid-laden vesicles. Moderate to severe steatosis can be identified with high accuracy (AUROC 0.93)[24]. However, the ability to detect steatosis in patients with NASH is limited by the presence of advanced fibrosis, with ultrasonography being less sensitive for steatosis in this patient group than in patients without advanced fibrosis[25]. It is also limited by both interobserver and intraobserver variability.

Controlled attenuation parameter. The controlled attenuation parameter (CAP) is a method for grading steatosis by measuring the degree of ultrasound attenuation by hepatic fat using a process based on simultaneous transient elastography[26]. Results are measured in dB/m over a range of 100–400 dB/m. CAP is now available with both M and XL probes, which cater to patients with different body builds. In a meta-analysis of 2,735 patients (only 20% of whom had NAFLD), the AUROCs were 0.82 for the diagnosis of any steatosis versus no steatosis and 0.87 for grade 2 or 3 steatosis versus grade 0 or 1 steatosis[26].

MRI. The MRI-estimated proton density fat fraction (MRI-PDFF) is an imaging-based biomarker that enables fat mapping of the entire liver. MRI-PDFF maps can be generated within seconds and can be analysed after minimal training to recognizing liver segments and avoid artefacts. This technology is more accurate than CAP in detecting all grades of steatosis in patients with NAFLD[27] (AUROC 0.99). Liver fat content estimated by MRI-PDFF correlates with that measured by magnetic resonance spectroscopy (MRS) and is more sensitive than the histology-determined steatosis grade in quantifying changes in the liver fat content over time[28].

MRS-PDFF provides a biochemical measure of liver fat in small regions of interest. MRS-PDFF is a research tool requiring special coils, is time consuming, is not routinely available and will unlikely be used in clinical practice because of the complexities of its logistics and the lack of required expertise at most clinical imaging centres[29]. Full sensitivity and specificity values for detection of liver fat of varying extents by MRS are given in TABLE 1.

Summary and recommendations

Abdominal ultrasonography is the most commonly used method to diagnose fatty liver in routine clinical settings because of its wide availability and low cost relative to MRI-PDFF and MRS. CAP measurement by transient elastography (FibroScan) is a more objective alternative to conventional ultrasonography. However, its responsiveness to changes in steatosis has not been established, making its use as an outcome measure in clinical trials premature. MRI-PDFF is highly accurate

Table 1 | **Noninvasive tests of hepatic steatosis**

Test	Description	Accuracy	Reproducibility	Responsiveness	Feasibility	Limitations
Blood biomarkers and panels						
Fatty liver index	BMI, WC, triglycerides and GGT	• AUROC 0.84 (Sn 87%, Sp 64%) • Cannot distinguish between steatosis grades	Not tested but should be fully reproducible	Moderate	High, as common parameters involved; also prognostic	Suboptimal gold standard (based on USG only)
Hepatic steatosis index	AST:ALT ratio, BMI, female sex and DM	• AUROC 0.81 (Sn 93%, Sp 92%) • Cannot distinguish between steatosis grades	Reproducible	Weak	High, as common parameters involved	Suboptimal gold standard (based on USG only)
NAFLD liver fat score	MetS, type 2 DM, fS-insulin, fS-AST and AST:ALT ratio	AUROC 0.86–0.87 (Sn 86%, Sp 71%)	Reproducible	None	Intermediate (fasting serum insulin level required)	Insulin not a routine test, hence limited availability
SteatoTest	Six components of the FibroTest–ActiTest plus BMI, cholesterol, triglycerides and glucose adjusted for age and sex	• AUROC 0.79–0.80 (Sn 85–100%, Sp 83–100%) • Cannot distinguish between steatosis grades	Reproducible	NA	Intermediate, as proprietary formula involved	FibroTest–ActiTest not available in all regions, hence limited availability; high cost
NAFLD ridge score	ALT, HDL-C, triglycerides, HbA_{1c}, WBC and hypertension	• AUROC 0.87 (Sn 92%, Sp 90%) • Cannot distinguish between steatosis grades	Reproducible	NA	Limited to research setting	Somewhat low positive predictive value (69%)
Imaging biomarkers						
USG	The echogenicity, or brightness, of tissue depends on the degree of beam scattering by the tissue (fat deposition in tissue accentuates scattering)	AUROC 0.93 for the diagnosis of steatosis (Sn 60–80%, Sp 80–100%)	Reliability: kappa statistics ranging from 0.54 to 0.92 for intrarater reliability and from 0.44 to 1.00 for interrater reliability	NA	• Easy to perform and interpret • No radiation • Available in extremely high numbers across medical centres across the world • Low cost	• Low sensitivity for mild steatosis • Operator-dependent • Reduction of Sn and Sp in patients who are obese and those with advanced fibrosis
Controlled attenuation parameter	Measurement of the degree of ultrasound attenuation by hepatic fat using a process based on simultaneous TE	• AUROC 0.82 for diagnosing any steatosis (Sn 69%, Sp 82%) • AUROC 0.86 for diagnosing stage 2 and stage 3 steatosis (Sn 77%, Sp 81%) • AUROC 0.88 for diagnosing stage 3 steatosis (Sn 88%, Sp 78%)	Concordance correlation coefficient 0.82	NA	• Immediate assessment of steatosis • Ambulatory clinic setting • Simultaneous liver stiffness measurement • Failure rate <10%	Does not reliably differentiate between steatosis grades
MRI-PDFF	PDFF measurement is an option that can be added to MRI scanners to quantitatively assess steatosis	AUROC 0.99 for diagnosing any steatosis (Sn 96%, Sp 100%, PPV 1.00, NPV 0.70)	ICC > 0.90	NA	• Not affected by obesity • Simultaneous MRI for liver architecture and carcinoma and MRS for steatosis	• Costly • Time consuming • Requires MRI facility • Might be inaccurate in acute inflammation or iron overload • Cannot be used in some patients with implantable devices

Table 1 (cont.) | **Noninvasive tests of hepatic steatosis**

Test	Description	Accuracy	Reproducibility	Responsiveness	Feasibility	Limitations
Imaging biomarkers (cont.)						
MRS	• Assesses liver triglyceride content • Provides a collection of spectra for signal fat fraction estimation, which requires a proper acquisition technique in order to estimate the fat	• Sn 89% and Sp 92% for diagnosis of liver fat with a threshold of 0–5% fat • Sn 83% and Sp 94% for diagnosis of 10% liver fat • Sn 73% and Sp 96% for diagnosis of liver fat > 30%	Very high with ICC 99.8%	NA	The absolute liver fat concentration can be directly measured, and very small amounts of liver fat (as low as 0.5%) can be detected and quantified	• Complex and time-consuming data analysis • Data collection occurs from a small portion of the liver (within a voxel ≤ 3 cm × 3 cm × 3 cm), which might be subject to sampling error

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under the receiver-operating characteristics curve; DM, diabetes mellitus; fS, fasting serum; GGT, gamma-glutamyltransferase; HbA_{1c}, haemoglobin A_{1c}; HDL-C, HDL cholesterol; ICC, intraclass correlation coefficient; MetS, metabolic syndrome; MRS, magnetic resonance spectroscopy; NA, not applicable; NPV, negative predictive value; PDFF, proton density fat fraction; PPV, positive predictive value; Sn, sensitivity; Sp, specificity; TE, transient elastography; USG, ultrasonography; WBC, white blood cell count; WC, waist circumference.

and reproducible and can be considered the gold standard for steatosis measurement. It can be used in clinical trials to detect steatosis changes, but its application in routine clinical practice is limited by cost and availability. The performance of blood biomarkers and panels is modest compared with imaging biomarkers, but these instruments can be readily applied in retrospective databases for large epidemiological studies.

Diagnosis of NASH

NASH is associated with faster fibrosis progression than NAFL and is currently the main target for pharmacological treatment. Patients with NASH are more likely to develop cirrhosis and die from cardiovascular and liver-related causes, although the prognostic importance of NASH is attenuated after adjustment for fibrosis stage[14]. This finding could be because of the dynamic nature of NAFLD; a snapshot view of disease activity might not be very informative. The diagnosis of NASH depends on liver biopsy and is also limited by interobserver variability among pathologists. In 2014, Bedossa and colleagues proposed the Fatty Liver Inhibition of Progression (FLIP) algorithm to improve the consistency of NASH diagnosis on the basis of histological steatosis, activity and fibrosis scores[30]. Instead of using global assessment by pathologists, the FLIP algorithm requires pathologists to follow standard criteria to score individual histological features and diagnose NASH.

Blood biomarkers and panels

The pathogenesis of liver injury in NASH is complex and involves numerous hormonal, cellular and molecular disturbances[9]. This intricacy has led to the examination of a multitude of potential NASH biomarkers that reflect underlying disease pathways, including hepatocellular apoptosis, inflammation, oxidative stress and abnormal adipokine signalling (TABLE 2). Serum aminotransaminase levels remain the most commonly used blood parameter for assessment of chronic liver disease, including NASH; however, ALT levels in isolation are poorly predictive of NASH and correlate best with hepatic triglyceride levels[31,32].

Individual blood biomarkers. Hepatocyte death via apoptosis or necroptosis is increased in NASH compared with simple steatosis and leads to the release of cytokeratin 18 (CK18; also known as KRT18) fragments. CK18 is the major intermediate filament protein within hepatocytes and is cleaved during initiation of cell death, which leads to extracellular release[33]. Serum levels of CK18 fragments and total length CK18 can be measured by an M30 and M65 antibody enzyme-linked immunosorbent assay (ELISA), with M30 levels correlating with liver inflammation and ballooning in patients with NAFLD and parallel histological improvement over time[34]. Nevertheless, there is considerable overlap of CK18 levels between patients with and without NASH; a meta-analysis of 11 studies found a pooled sensitivity and specificity for NASH of 66% and 82%, respectively, suggesting that it is not sufficiently accurate for clinical use[35]. To increase diagnostic utility, CK18 has been combined with serum levels of apoptosis-mediating surface antigen FAS (sFAS), which is involved in the activation of the extrinsic apoptosis pathway in hepatocytes[36]. A small study demonstrated that the combination of M30 and sFAS levels had reasonable to excellent accuracy (AUROC of 0.79–0.93); however, further validation studies are required[36].

Inflammation is one of the histological hallmarks of NASH, and thus serum levels of many inflammatory markers and mediators, including C-reactive protein (CRP), TNF, IL-6 and IL-8, IL-1 receptor antagonist protein (IL-1RA) and CXC-chemokine 10 (CXCL10), have been examined as diagnostic markers. As NASH is associated with an underlying inflammatory metabolic state, these markers might not be specific for liver inflammation. Notably, a study of 648 patients with biopsy-proven NAFLD that tested the diagnostic utility of 32 plasma biomarkers, including many inflammatory markers, found that levels of IL-8, soluble IL-1R type 1 (IL-1R1), total plasminogen activator inhibitor 1 (PAI1) and activated PAI1 (aPAI1) were associated with NASH. However, only aPAI1 remained predictive of NASH after adjustment for clinical and metabolic factors[37]. aPAI1 is an inhibitor of fibrinolysis and might

Table 2 | **Biochemical blood markers of NASH**

Blood biomarkers and panels	Candidates	Advantages	Disadvantages
Apoptosis markers	• CK18 fragments • Total cytokeratin • sFAS	• CK18 is the most well-validated blood biomarker • Assays available commercially • Correlate with histological improvement over time	• Limited accuracy in isolation • Optimal cut-offs uncertain • Poor sensitivity
Inflammatory markers	• CRP • TNF • IL-8 • CXCL10	• Correlate with inflammatory activity in NASH • Assays for most markers are available commercially	• Not validated as diagnostic markers • Might be influenced by systemic inflammation
Lipid oxidation products	• 11-HETE • 9-HODE, 13-HODE • 13-oxo-ODE • LA–13-HODE (oxNASH score) • 11,12-diHETrE	Good to excellent accuracy in small studies	• Need further validation • Require mass spectroscopy
Adipocytokines and hormones	• Adiponectin • Leptin • Resistin • Visfatin • RBP4 • FABP4 • FGF21	• Majority are commercialized assays • FGF21 dynamic to changes in NAFLD over time	• Limited accuracy in isolation • Mostly validated only in bariatric populations
Lysosomal enzymes	Cathepsin D	• Commercial assay available • Dynamic to changes in NAFLD over time	• Interpretation different in adults and children • Limited validation
Combined panels	• NASHTest • NASH Diagnostics Panel	• Available commercially • Moderate to high degree of accuracy • Reliable	• Majority of validation is in bariatric populations • High cost • Unknown whether dynamic to changes in histology

CK18, cytokeratin 18; CRP, C-reactive protein; CXCL10, CXC-chemokine ligand 10; diHETrE, dihydroxy-eicosatrienoic acid; FABP4, fatty acid-binding protein 4; FGF21, fibroblast growth factor 21; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; LA, linoleic acid; oxo-ODE, oxo-octadecadienoic acid; RBP4, retinol-binding protein 4; sFAS, serum levels of apoptosis-mediating surface antigen FAS.

link NASH with increased cardiovascular risk through its effect on coagulation. aPAI1 is unlikely to be sufficiently diagnostic in isolation, and further studies examining its diagnostic utility in combination with other markers are awaited.

Oxidative stress is one of the principal pathogenic mechanisms of liver injury in NASH and leads to lipid oxidation and the generation of serum products that can be measured[38]. Lipidomic studies utilizing mass spectroscopy have found lipid oxidation products to be associated with NASH, including products of arachidonic acid oxidation (11-hydroxyeicosatetraenoic acid (11-HETE)) and products of linoleic acid oxidation (9 and 13 hydroxyoctadecadienoic acid (HODE) and 9 and 13 oxo-octadecadienoic acids (oxo-ODE))[39,40]. The combination of linoleic acid:13-HODE ratio plus age, BMI and AST forms the oxNASH score, which has been validated as providing reasonable diagnostic accuracy for NASH (AUROC 0.74–0.83)[40]. Nevertheless, the cost of and need for specialized equipment and specimen processing currently limit widespread application of lipid oxidation products as a diagnostic tool for NASH.

A range of hormones involved in lipid and glucose metabolism are disturbed in NASH, including adipokines (adiponectin, leptin, resistin, visfatin (also known as NAMPT), retinol-binding protein 4 (RBP4) and fatty acid-binding protein 4 (FABP4)) and liver-derived hormones, including fibroblast growth factor 21 (FGF21)[41]. These hormones might be involved in the pathogenesis of liver injury; however, serum levels could also reflect associated metabolic abnormalities such as visceral adiposity and thus might not be specific for NASH. Consequently, there are conflicting studies regarding the independent association between adipokines and NASH[37,42,43]. By contrast, a meta-analysis published in 2017 demonstrated a consistent association between serum FGF21 levels and NASH. However, this biomarker was only modestly sensitive and specific, with pooled values of 62% and 78%, respectively[44].

Ferritin is an acute-phase reactant that is commonly increased in patients with NAFLD and metabolic syndrome. Previous studies have shown an association between hyperferritinaemia and advanced fibrosis in patients with NAFLD[45]. The addition of non-proprietary and/or a specific fibrosis biomarkers might also increase the diagnostic accuracy of this reactant for NASH[46,47]. In one study of 405 patients with NAFLD, the addition of AST, BMI, platelet count, diabetes status and hypertension presence to serum ferritin levels increased the AUROC for the diagnosis of NASH to 0.81, in comparison with an AUROC of 0.62 when ferritin was used alone[37]. In another study, a combined

panel of serum ferritin, fasting insulin and type IV collagen 7S achieved an AUROC of 0.78 for the diagnosis of NASH[38].

Biomarker panels. Owing to the modest accuracy of individual markers of NASH, combinations of markers have been examined to increase diagnostic utility. The NASHTest (Biopredictive) is a proprietary algorithm consisting of a combination of age, gender, height, weight and serum levels of triglyceride, cholesterol, α_2m, apolipoprotein AI, haptoglobin, GGT, ALT, AST and total bilirubin[48]. The accuracy of the NASHTest in training and validation cohorts totalling 257 patients was between 0.69 and 0.79. Other combination panels have frequently included CK18 quantification; Younossi and colleagues examined the levels of a combination of cleaved and intact CK18, adiponectin and resistin in 101 patients and found AUROC values between 0.73 and 0.91 for the diagnosis of NASH. They subsequently developed the NASH Diagnostic Panel (comprising diabetes mellitus presence, sex, BMI and serum levels of triglyceride, CK18 fragments and total CK18), which could diagnose NASH with an AUROC of 0.81 (REF.[49]). Both these panels were developed in somewhat small numbers of patients with obesity, and thus further validation is required. Another panel utilizing the combination of serum CK18 and FGF21 levels in a step-wise approach found high specificity and sensitivity values of >90% for NASH when applying optimal cut-off values, but this was at the cost of indeterminate values in ~70% of patients[42]. Plasma levels of cathepsin D (CTSD; a lysosomal enzyme) were found to have an accuracy of 0.998 for the prediction of NASH in a paediatric population when combined with plasma CK18 levels[50]. A subsequent study in adults found that serum CTSD levels in isolation had 84% accuracy for NASH; levels of CTSD fell substantially after weight loss surgery, suggesting that this marker is dynamic to treatment[51]. Confusingly, however, CTSD levels were high in adult patients with NASH but low in paediatric patients, underscoring the need for further validation.

Despite the large number of serum biomarkers and combination panels in the literature, there is a lack of independent validation, particularly in different ethnic groups or in non-bariatric surgery populations. Similarly, there is uncertainty regarding the optimum diagnostic cut-offs and a lack of knowledge regarding the influence of other factors that might affect test interpretation. Apart from CK18, there is limited assessment of the dynamic nature of biomarkers to determine whether they reflect changes in histology over time and thus can be used to monitor disease progression or treatment efficacy. Lastly, a NASH biomarker should demonstrate cost-effectiveness and directly influence patient management before widespread adoption into clinical practice.

Imaging biomarkers

Routine imaging by abdominal ultrasonography, CT or MRI is unable to distinguish between NAFL and NASH. A number of MRI-based techniques have been tried in animal and pilot human studies for the diagnosis of NASH. Super paramagnetic iron oxide MRI can detect Kupffer cell uptake function, which is impaired in animals and humans with NASH, but the current protocol requires repeated scanning over 72 h and thus poses logistical difficulties[52]. When the hepatobiliary contrast gadoxetic acid is used, patients with NASH have increased liver enhancement on MRI[53]. Hepatocyte membrane turnover and intracellular ATP can be measured by phosphorus MRS and are altered in patients with NASH[54]. In particular, the alpha-nucleotide triphosphate:total phosphate ratio had an AUROC of 0.71 for diagnosing NASH. Liver stiffness measurement by magnetic resonance elastography (MRE, described in detail in the diagnosis of fibrosis and cirrhosis section) is also increased in NASH, but the diagnostic accuracy needs to be evaluated in larger cohorts with careful adjustment for fibrosis stage[55].

Summary and recommendations

CK18 is the most extensively evaluated test for NASH diagnosis, but overall accuracy is moderate at best. Although other biomarkers or panels might hold promise, most have not been independently validated. At present, none of the NASH biomarkers are ready for routine clinical use. However, active research in this field will further inform practice. The use of different NASH biomarkers in clinical trials depends on the mechanism of action of the study drugs. For example, cell death markers might be more relevant for agents targeting hepatocyte apoptosis[56]. Biomarkers well suited for assessing metabolic changes, apoptosis or cell death, inflammation or fibrogenesis are therefore of greatest relevance.

Diagnosis of fibrosis and cirrhosis

Cirrhosis is the final common pathway of chronic liver diseases. Once cirrhosis sets in, a patient is at risk of developing portal hypertension, liver decompensation and HCC[57]. A meta-analysis published in 2017 further confirms that fibrosis stage is associated with all-cause mortality in a dose-dependent manner, with increased risk first apparent in patients with F2 fibrosis[58]. Because of the close association between fibrosis stage and clinical outcomes, one can argue that effective treatments for NASH must improve fibrosis or at least prevent its progression.

Non-proprietary biomarkers and panels

Most non-proprietary biomarkers of fibrosis do not directly measure fibrogenesis or fibrinolysis. They often represent biological processes associated with risk factors of fibrosis and are commonly performed and inexpensive. However, such indirect biomarkers are in general less accurate than biomarkers directly measuring fibrogenesis or fibrinolysis. To compensate for the reduced accuracy, most investigators use a panel of biomarkers (TABLE 3). On the whole, although these panels have modest accuracy, many have negative predictive values of over 90% in excluding advanced fibrosis or cirrhosis at the proposed cut-offs. They are, however, less reliable than other methods in discriminating among fibrosis stages.

The AST:ALT ratio and AST:platelet ratio index (APRI) were initially derived from chronic hepatitis C cohorts[59,60]. They are simple to calculate but have low accuracy in diagnosing advanced fibrosis and

Table 3 | **Noninvasive tests of liver fibrosis and cirrhosis**

Test	Description	Accuracy	Reproducibility	Responsiveness	Feasibility	Limitations
AST:ALT ratio	AST and ALT	AUROC 0.66–0.74 for F3 fibrosis (Sn 40%, Sp 80%)	Not tested, but aminotransferases can change rapidly over time	NA	High, as common parameters involved	Modest accuracy
AST:platelet ratio index	AST and platelet count	AUROC 0.74 for F3 fibrosis (Sn 65%, Sp 72%)	Not tested, but aminotransferases can change rapidly over time	Modest	High, as common parameters involved	Modest accuracy
Fibrosis-4 index	Age, AST, ALT and platelet count	AUROC 0.80 for F3 fibrosis (Sn 65%, Sp 97%; by dual cut-offs)	Not tested, but aminotransferases can change rapidly over time	Modest	High, as common parameters involved	NA
NAFLD fibrosis score	Age, BMI, impaired fasting glucose and/or diabetes, AST, ALT, platelet count and albumin	AUROC 0.75–0.82 for F3 fibrosis (Sn 73–82%, Sp 96–98%; by dual cut-offs)	Not tested, but aminotransferases can change rapidly over time	Modest	High, as common parameters involved	Interpretation of BMI might differ across different ethnic groups
BARD score	AST, ALT, BMI and diabetes	AUROC 0.69–0.81 for F3 fibrosis (Sn 62%, Sp 66%)	Not tested, but aminotransferases can change rapidly over time	NA	High, as common parameters involved	Interpretation of BMI might differ across different ethnic groups

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under the receiver-operating characteristics curve; BARD score, calculated from BMI, AST:ALT ratio and diabetes mellitus presence; NA, not applicable; Sn, sensitivity; Sp, specificity.

cirrhosis in patients with NAFLD (AUROC 0.66–0.74 and AUROC 0.74 for the discrimination of F3 fibrosis, respectively). The Fibrosis-4 (FIB-4) index comprises age, AST, ALT and platelet count and is calculated with a more complicated formula; it has moderate accuracy (AUROC 0.83 for detecting F3 fibrosis), which is comparable to that of the NAFLD fibrosis score[61].

The NAFLD fibrosis score was specifically derived and validated in patients with biopsy-proven NAFLD[62]. The formula consists of age, BMI, presence of impaired fasting glucose or diabetes mellitus, AST:ALT ratio, platelet count and serum albumin levels. It has an AUROC of 0.82 in detecting F3 fibrosis and has been more extensively validated than the other scores[63]. Importantly, the score has been shown to predict liver decompensation and mortality in patients with NAFLD[64].

The BARD score (calculated from BMI, AST:ALT ratio and diabetes mellitus presence) was also derived from a large American NAFLD cohort; it has moderate accuracy in detecting F3 fibrosis in subsequent validation studies (AUROC 0.69–0.81)[65].

Although these scores are less accurate than specific fibrosis markers, they have high applicability, as the components are all readily available to clinicians. Although some scores require complex calculations, the availability of online calculators and corresponding apps has rendered this hurdle less relevant. Besides, these scores have high negative predictive value in excluding advanced fibrosis and liver-related events and are therefore reasonable screening tools particularly when more sophisticated tests are unavailable[66].

Specific fibrosis markers

Specific fibrosis markers reflect fibrogenesis and/or fibrinolysis. Although there is overlap in values across fibrosis stages, some proprietary panels (such as FibroTest and FibroMeter) provide the exact predicted fibrosis stage in the report. Liver fibrosis develops through the hepatic deposition of extracellular matrix (ECM), of which hyaluronic acid (HA), a non-proteoglycan polysaccharide, is a major component[67] (TABLE 4). When used alone, serum HA level has an AUROC of 0.87 for F2 fibrosis and 0.92 for cirrhosis[68]. Other major components of the ECM include proteoglycans (such as heparan sulfate, chondroitin sulfate and keratan sulfate), collagen, elastin, fibronectin and laminin. Procollagen III amino-terminal peptide (PIIINP) in the serum is derived from the synthesis of new type III collagen or from the degradation of existing type III collagen fibrils; PIIINP levels alone are not a good diagnostic marker for liver fibrosis in NAFLD or alcoholic liver disease[69]. A neo-epitope-specific competitive ELISA for PIIINP, that is, Pro-C3, reflects true formation of type III collagen, as it measures the propeptide cleaved off from the intact collagen molecule and is specific for liver collagen[70]. Raised serum Pro-C3 levels correlate with key components of NASH and fibrosis[71].

Circulating levels of tissue inhibitor of metalloproteinases 1 (TIMP1), which regulates matrix metalloproteinases and is responsible for the ECM composition and wound healing, reflect alterations in tissue matrix remodelling during hepatic fibrogenesis and fibrinolysis[72]. TIMP1 has excellent diagnostic accuracy (AUROC 0.97) to differentiate patients who are obese and have NASH from age-matched healthy control individuals[73]. Laminin is the most abundant non-collagenous glycoprotein in basement membranes. Serum laminin level is able to diagnose fibrosis in patients with NAFLD to a satisfactory level of accuracy (AUROC 0.87)[74]. Although the biomarkers discussed in this section are promising, their cost and availability have limited their adoption in routine practice.

Table 4 | **Specific fibrosis markers and panels**

Test	Description	Accuracy	Reproducibility	Responsiveness	Feasibility	Limitations
Specific fibrosis markers						
Hyaluronic acid	Important structural role in extracellular matrix	AUROC 0.99 for F4 fibrosis (Sn 98%, Sp 100%)	Not tested	NA	Important role in the common pathway of fibrosis of different aetiologies; included in a few blood panels	Cannot be used alone
PIIINP	Direct measurement of the synthesis and degradation of existing type III collagen fibrils	NA	Not tested	NA	Important role in the common pathway of fibrosis of different aetiologies; included in a few blood panels	Cannot be used alone
Pro-C3	Reflects true formation of type III collagen	NA	Not tested	NA	Correlated well with NAFLD activity score	Not well studied in other chronic liver diseases
TIMP1	Reflects tissue matrix remodelling during fibrogenesis and fibrolysis	AUROC 0.97 for NASH (Sn 97%, Sp 100%)	Not tested	NA	Excellent diagnostic accuracy (to differentiate NASH from control); included in a few blood panels	Cannot be used alone in chronic liver diseases other than NAFLD
Laminin	Non-collagenous glycoprotein in basement membranes	AUROC 0.87 for NAFLD with fibrosis (Sn 82%, Sp 89%)	Not tested	NA	Important role in the common pathway of fibrosis of different aetiologies	Data from small studies only
Specific fibrosis panels						
ELF	PIIINP, hyaluronic acid, TIMP1	AUROC 0.92 for F1 fibrosis (Sn 88%, Sp 81%), 0.98 for F2 fibrosis (Sn 94%, Sp 93%) and 0.99 for F3 fibrosis (Sn 100%, Sp 98%)	Good	NA	Good prognostic factor for clinical outcomes in patients with chronic liver diseases; similar results by using fresh blood or cryopreserved blood	Not sensitive for early stages of fibrosis; age, low $CD4^+$ T cell count and other factors can affect ELF score results
FibroTest	GGT, total bilirubin, α_2m, apolipoprotein AI and haptoglobin	Non-binary AUROC for fibrosis 0.88	Good	NA	Useful in different chronic liver disease; accurate in patients with overweight or obesity	Suboptimal for early-stage fibrosis
FibroMeter NAFLD	body weight, prothrombin index, ALT, AST, ferritin and fasting glucose	AUROC 0.76 for F2 fibrosis (Sn 22%, Sp 97%), 0.77 for F3 fibrosis (Sn 27%, Sp 95%)	Good	NA	Accurate for severe fibrosis in different liver diseases	High cost

α_2m, α_2-macroglobulin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under the receiver-operating characteristics curve; ELF, Enhanced Liver Fibrosis; GGT, gamma-glutamyltransferase; NA, not applicable; PIIINP, procollagen III amino-terminal peptide; Pro-C3, neo-epitope-specific competitive enzyme-linked immunosorbent assay for PIIINP; Sn, sensitivity; Sp, specificity; TIMP1, tissue inhibitor of metalloproteinases 1.

The Enhanced Liver Fibrosis (ELF) panel is an algorithm consisting of three specific fibrosis markers discussed above: PIIINP, HA and TIMP1 (REF.[75]). ELF was found to be accurate to predict advanced fibrosis in adult and paediatric patients with NAFLD (AUROC 0.93 and 0.99, respectively)[76,77]. FibroTest (Biopredictive; or known as Fibrosure in the United States) consists of five components: serum levels of GGT, total bilirubin, α_2m, apolipoprotein AI and haptoglobin[78]. FibroTest was found to be better than BARD and FIB-4 at predicting fibrosis in patients with NAFLD (AUROC 0.88 for any fibrosis)[79]. FibroMeter NAFLD (Echosens, Paris, France) is an index composed of body weight, prothrombin index and serum levels of ALT, AST, ferritin and fasting glucose. In two studies conducted in Europe and Asia, FibroMeter NAFLD outperformed other serum tests for the diagnosis of fibrosis[80,81]. The FibroMeter vibration-controlled transient elastography algorithm (combining FibroMeter NAFLD and liver stiffness measurement) might improve the diagnosis of F3–F4 fibrosis versus F0–F2 fibrosis[81]. Hepascore, composed of age, sex and serum levels of bilirubin, GGT, HA and α_2m, has similarly satisfactory accuracy as other serum formulae (AUROC 0.82) to diagnose advanced fibrosis (stage F3–F4) in patients with NAFLD[82].

Despite slight improvement in diagnostic accuracy over non-proprietary biomarkers, the limited availability of these specific fibrosis markers and the cost of using proprietary formulae might limit their wider application.

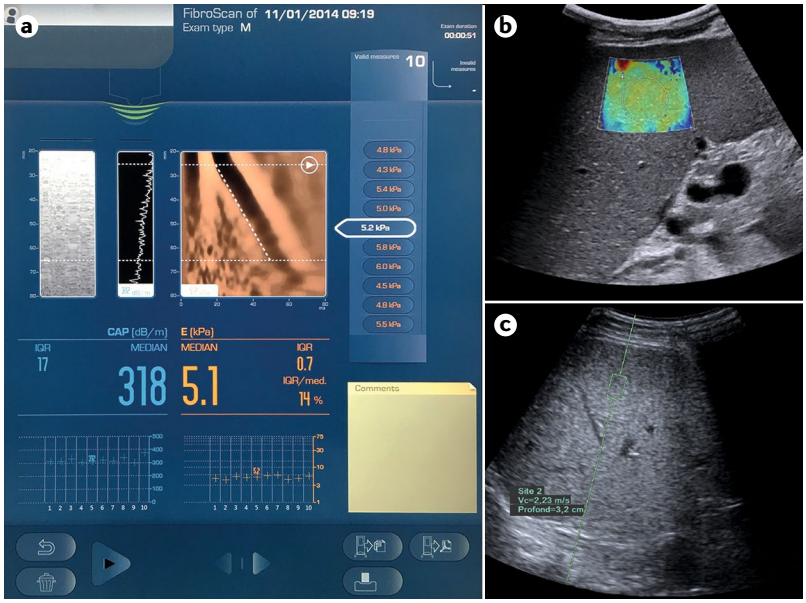

Fig. 2 | **Ultrasound-based measurement of liver stiffness or elasticity.** Transient elastography measures the controlled attenuation parameter and liver stiffness simultaneously and allows assessment of both hepatic steatosis and fibrosis (part **a**). Shear wave elastography (part **b**) and acoustic radiation force impulse (part **c**) are incorporated in an ultrasonography machine and thus can be performed together with structural examination and hepatocellular carcinoma screening. Visualization of the liver parenchyma might also increase the success rate of measurement, especially in patients who are obese. Images in parts **b** and **c** courtesy of C. Cassinotto (Department of Radiology, St-Eloi University Hospital, Montpellier, France) and N. Frulio (Department of Radiology, Haut Leveque Hospital CHU Bordeaux, Pessac, France), respectively.

Imaging biomarkers

FibroScan. FibroScan measures the velocity of an elastic shear wave propagating through the liver[29] (FIG. 2; TABLE 5). This velocity is directly related to tissue stiffness, which in turn is related to the degree of fibrosis; the stiffer the tissue, the faster the shear wave propagates. The measurement procedure is considered to have failed when no value is obtained after ten attempts. An examination is considered valid if ≥10 valid measurements are obtained and the interquartile range (which reflects the variability of measurements) is <30% of the median liver stiffness measurement. The results are expressed in kilopascals and range from 1.5 to 75 kPa, with normal values near 5 kPa. The summary AUROC values using FibroScan M and XL probes for diagnosing advanced fibrosis are 0.88 and 0.85, respectively[83].The XL probe was designed to cater to patients who are obese and produces similar diagnostic accuracy as the M probe in patients who are not obese[84,85].

Although FibroScan has excellent negative predictive value to exclude advanced fibrosis, its positive predictive value to rule in advanced fibrosis or cirrhosis is modest (TABLE 3). As such, a number of studies have tried to combine FibroScan and a non-proprietary formula such as the NAFLD fibrosis score to improve the diagnostic accuracy[86–88]. Potential applications of combined tests include performing both tests and considering the diagnosis confirmed if they provide concordant results and reserving the second test for when results from the first test are inconclusive. In most situations, the second approach is more attractive because FibroScan already has a high negative predictive value; a second test will add little information for patients with low liver stiffness.

Point shear wave elastography. Point shear wave elastography (pSWE), also known as acoustic radiation force impulse (ARFI), involves mechanical excitation of tissue using short-duration acoustic pulses that propagate shear waves and generate localized, micrometre-scale displacements in tissue[89] (FIG. 2). The exact location where measurements are obtained can be selected by the operator. A major advantage of pSWE is that it can be easily implemented on modified commercial ultrasonography machines. Therefore, liver fibrosis and the liver parenchyma can be assessed during the same examination. Values obtained with pSWE are expressed in m/s and have a narrow range (0.5–4.4 m/s), which limits the definitions of cut-off values for discriminating certain fibrosis stages and thus for making management decisions. For instance, pSWE performance is better for severe fibrosis and cirrhosis than for less severe fibrosis[90]. However, pSWE has not been extensively evaluated, and studies are needed to determine the effects of the narrow measurement range and discrepancies between right and left liver lobe readings.

2D shear wave elastography. 2D shear wave elastography (2D-SWE) is based on the combination of a radiation force induced in tissues by focused ultrasonic beams and a very high frame rate ultrasonography imaging sequence capable of catching in real time the transient propagation of resulting shear waves[91] (FIG. 2). The size of the region of interest can be chosen by the operator. 2D-SWE also has the advantage of being implemented on a commercially available ultrasonography machine, with results expressed either in m/s or in kilopascals across a wide range of values (2–150 kPa)[90]. 2D-SWE has a higher accuracy than pSWE for diagnosis of F2 fibrosis (AUROC 0.85–0.92 versus AUROC 0.70–0.83).

Magnetic resonance elastography. MRE uses a modified phase-contrast method to image the propagation of the shear wave in the liver parenchyma. It can assess the entire liver, irrespective of body habitus, and mechanical parameters for shear stiffness have been standardized[92]. A meta-analysis of nine studies comprising 232 patients with NAFLD found that MRE detected fibrosis with a high level of accuracy, independent of liver inflammation and BMI (unlike FibroScan), with AUROC values of 0.86–0.91 for all stages of fibrosis[93]. This technique is more accurate than FibroScan in detecting F2 fibrosis (AUROC 0.86–0.89 versus AUROC 0.84) and F4 fibrosis (AUROC 0.88–0.97 versus AUROC 0.95)[27,94]. However, its wider application is limited by cost and availability. In particular, these drawbacks make it unlikely that MRE can be applied as a screening test.

Multiparametric MRI. In the past few years, a multiparametric magnetic resonance technique (LiverMultiScan, Perspectum Diagnostics, Oxford, UK) has been established that includes T1 mapping for fibrosis and inflammation imaging, T2 mapping for liver iron

Table 5 | **Imaging biomarkers for liver fibrosis**

Test	Description	Accuracy	Reproducibility	Responsiveness	Feasibility	Limitations
FibroScan (TE)	• Mechanically induced impulse • Quantitative measurement of shear wave speed • Two probes: M and XL (for patients with BMI > 30 kg/m²)	• AUROC 0.84 for F2 fibrosis with the M probe (Sn 79%, Sp 76%) • AUROC 0.93 for F3 fibrosis with the M probe (Sn 91%, Sp 75%, PPV 52%, NPV 97%) • AUROC 0.95 for F4 fibrosis with the M probe (Sn 92%, Sp 88%) • AUROC 0.80–0.85 for F2 fibrosis with the XL probe (Sn 76%, Sp 65%, PPV 65%, NPV 79%) • AUROC 0.84–0.90 for F3 fibrosis with the XL probe (Sn 75%, Sp 74%, PPV 0.59, NPV 0.89) • AUROC 0.91–0.95 for F4 fibrosis with the XL probe (Sn 88%, Sp 82%, PPV 40%, NPV 98%)	ICC > 0.90	Limited data	• Short processing time (<10 minutes) • Ambulatory clinic setting • Immediacy of results • 0–10% of measurements are failures	• Requires fasting for 2 hours • Requires a dedicated device
pSWE (ARFI)	• Ultrasound-induced focused radiation force impulse at death • Quantitative measurement of shear wave speed	• AUROC 0.70–0.83 for F2 fibrosis (Sn 56–90%, Sp 36–90%) • AUROC 0.74–0.97 for F3 fibrosis (Sn 59–90%, Sp 63–90%) • AUROC 0.78–0.89 for F4 fibrosis (Sn 44–90%, Sp 67–90%)	ICC 0.86–0.95	Limited data	• Implemented on a regular ultrasonography machine • Enables simultaneous sonographic imaging of the liver	• Requires fasting for 2 hours • Quality criteria not well defined
2D–3D SWE	• Ultrasound-induced radiation force focus swept over depth faster than shear wave speed to create a Mach cone • Quantitative measurement of shear wave speed	• AUROC 0.85–0.92 for F2 fibrosis (Sn 85%, Sp 94%, PPV 94%, NPV 85%) • AUROC 0.88–0.95 for F3 fibrosis (Sn 90%, Sp 92%, PPV 88%, NPV 93%) • AUROC 0.97 for F4 fibrosis (Sn 100%, Sp 86%, PPV 55%, NPV 100%)	ICC 0.92–0.95	Limited data	• Implemented on a regular ultrasonography machine • Enables simultaneous sonographic imaging of the liver	• Requires fasting for 2 hours • Experienced operators needed • Quality criteria not well defined
MRE	Uses a modified phase-contrast method to image the propagation of the shear wave in the liver parenchyma	• AUROC 0.86–0.89 for F2 fibrosis (Sn 73%, Sp 89%, PPV 84%, NPV 86%) • AUROC 0.89–0.96 for F3 fibrosis (Sn 86%, Sp 91%, PPV 71%, NPV 93%) • AUROC 0.88–0.97 for F4 fibrosis (Sn 87%, Sp 93%, PPV 53%, NPV 99%)	ICC 0.83–0.96	High concordance with histological severity and percentage collagen area in drug trials	• Implemented on a regular MRI machine • Examination of the whole liver	• Requires an MRI facility • Time consuming • Costly

Limitations of all imaging methods include confounding by infiltrative liver disease, liver congestion, acute hepatitis, liver inflammation and cholestasis. AUROC, area under the receiver-operating characteristics curve; ICC, intraclass correlation coefficient; MRE, magnetic resonance elastograph; NPV, negative predictive value; PPV, positive predictive value; pSWE, point shear wave elastography; Sn, sensitivity; Sp, specificity; SWE, shear wave elastography; TE, transient elastography.

quantification and ^{1}H-MRS for liver fat quantification[95]. The T1 measurements are adjusted for the iron level, as high iron levels in the presence of fibrosis can lead to 'pseudo-normal' T1 values. LiverMultiScan is a quick and noninvasive test that does not require injection of any intravenous contrast agent. In a study published in 2017, the AUROC using liver inflammation and fibrosis for the diagnosis of NAFLD-related cirrhosis was 0.85 (REF.[96]). Further validation studies are needed.

Summary and recommendations

For equipped centres, ultrasound-based elastography such as FibroScan and shear wave elastography has moderate to high accuracy in diagnosing advanced fibrosis or cirrhosis and can be used in routine clinical practice (TABLE 5). MRE has a higher success rate and accuracy than ultrasound-based technologies but is limited by cost and availability. However, it can be used in clinical trials to identify drugs with potential antifibrotic effects. If the imaging methods detailed are unavailable, blood biomarkers and clinical prediction rules are reasonable alternatives to rule out advanced fibrosis, although they are less accurate in discriminating across fibrosis stages.

Genetic biomarkers

Over the past few years, knowledge of the genetic component of NAFLD has grown exponentially, in part owing to genome-wide association studies and the advent of high-throughput omics technologies. The newly acquired genetic information has helped improve the understanding of disease pathogenesis, enabling novel strategies for early identification of at-risk patients to be envisioned. Nevertheless, incorporation of NAFLD genetic markers into routine clinical testing that can be used to dynamically assess the disease status and response to therapy has been slow, and the information yielded has had moderate utility. Here, we provide a critical appraisal of genetic and genomic markers in

Table 6 | **Genetic and genomic markers for noninvasive assessment of NAFLD risk and severity**

Biomarker (sample type)	Feasibility	Study population	Description	Validation	Accuracy	Limitations and reproducibility	Refs
Assessment of disease susceptibility							
SNP (DNA from peripheral blood)	LCT, HR and A	470 individuals with and without T2D; liver fat content measured using ^{1}H-MRS	The NAFLD liver fat score is a combination of routine clinical (T2D and MetS) and biochemical data (plasma insulin, ALT and AST) and rs738409 genotypes	Two-stage study (discovery and validation)	AUROC (prediction of liver fat content) 0.872 ± 0.020 (95% CI 0.84–0.91)	Addition of rs738409 to the score improved the accuracy of the prediction by only <1% (AUROC without genetic information 0.866 ± 0.020; 95% CI 0.83–0.90)	20
Cell-free ncRNAs (serum)	MCT, MR and A	275 patients with NAFLD and 190 healthy individuals; disease assessed by liver biopsy	Genome-wide profiling of circulating miRNAs; use of the predictive value of an miRNA panel (including miR-122, miR-1290, miR-192 and miR-7b)	Two-stage study (discovery and validation)	AUROC (prediction of NAFLD) 0.856 (95% CI 0.804–0.907); Sn 85.55% and Sp 73.30%	Poor specificity and limited cost-effectiveness for screening programmes	105
Composite panel of serum-derived omics data	MCT, MR and E	576 patients with morbid obesity enrolled in a bariatric surgery programme	Multicomponent score integrating variables from genomic (rs738409), phenomic (19 clinical variables) and proteomic data (including ACY1, SHBG, CTSZ, MET, GNS, LGALS3BP, CHL1 and SERPINC1)	Two-stage study (discovery and validation)	AUROC (prediction of NAFLD) 0.932 (95% CI 0.913–0.959) in the discovery set	• Limited cost-effectiveness for screening programmes • ELISA-based measurement of proteins in circulation that are not routinely used in the clinical setting	100
Assessment of disease severity							
SNP and circulating metabolites (DNA from peripheral blood and serum)	MCT, MR and E (MS-based method)	318 patients; disease severity assessed by liver biopsy	• The NASH Clinical score is a combination of laboratory tests (AST and fasting insulin) and rs738409 genotypes • The NASH ClinLipMet score is a combination of laboratory tests (AST and fasting insulin), circulating metabolites (glutamate, isoleucine, glycine, lyso-PC 16:0 and PE 40:6) and rs738409 genotypes	Two-stage study (discovery and validation)	• AUROC (prediction of NASH) 0.778 (95% CI 0.709–0.846) • AUROC (prediction of NASH) 0.866 (95% CI 0.820–0.913)	rs738409 genotypes correlate with NASH, but only measurement of saturated and mono-unsaturated lipids add value to the score	101
SNPs at multiple loci (DNA from peripheral blood)	LCT, HR and A	152 children who were obese and had NAFLD; disease severity assessed by liver biopsy	The genetic risk score of NASH includes a combination of four SNPs in different loci (including *PNPLA3*, *SOD2*, *KLF6* and *LPIN1*)	Single-stage study	AUROC (prediction of NASH) 0.75 (95% CI 0.67– 0.82)	Results are limited to children with overweight or obesity with elevated ALT levels and are from a pilot study that needs replication and validation	102
Cell-free ncRNA (serum); circulating miR-122 analysed alone	MCT, MR and A	96 participants enrolled in a case–control study; disease severity assessed by liver biopsy	Profiling of circulating miRNAs ($n = 84$); paired serum and liver biopsy sample analysis	Two-stage study (discovery and validation)	• AUROC (prediction of NASH) 0.714 (95% CI 0.524–0.861) • AUROC (prediction of fibrosis) 0.613 (95% CI 0.458–0.753)	Performance of miR-122 in predicting NASH is similar to that of other circulating biomarkers, including CK18	106

Table 6 (cont.) | **Genetic and genomic markers for noninvasive assessment of NAFLD risk and severity**

Biomarker (sample type)	Feasibility	Study population	Description	Validation	Accuracy	Limitations and reproducibility	Refs
Assessment of disease severity (cont.)							
Composite panel including cell-free ncRNAs (serum)	NA	198 participants enrolled in a case–control study; NAFLD assessed by liver biopsy	Combination score of miR122, miR192, miR21, ALT and CK18 Asp396 to differentiate between NAFL and NASH	Single-stage study	AUROC (prediction of NASH) 0.830 (95% CI 0.754–0.908)	• Addition of miRNAs to the score improved the accuracy of NASH prediction by only ~2% compared with CK18 Asp396 (AUROC 0.81 95% CI 0.725–0.888) • Data are from a pilot study that needs replication and validation	107
Cell-free DNA (circulating methylated *PPARG*)	HCT, MR and E	26 patients with NAFLD; disease severity assessed by liver biopsy	Assessment of plasma DNA methylation of *PPARG* gene promoter (two CpG dinucleotides assessed)	Single-stage study	AUROC (prediction for fibrosis) 0.91 (95% CI 0.80–1.00)	• Lack of comparison of NAFL versus NASH • Nonspecific for NAFLD (same performance in ALD); results are highly dependent on dying hepatocytes • Data are from a pilot study that needs replication and validation	109

A, affordable; ACY1, aminoacylase 1; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under the receiver-operating characteristics curve; CHL1, neural cell adhesion molecule L1-like protein; CK18, cytokeratin 18; CK18 Asp396, apoptosis-associated neoepitope cytokeratin 18 Asp396; CTSZ, cathepsin Z; E, expensive; ELISA, enzyme-linked immunosorbent assay; GNS, glucosamine (*N*-acetyl)-6-sulfatase; ^{1}H-MRS, proton magnetic resonance spectroscopy; HCT, high-complexity technology; HR, high reproducibility; LCT, low-complexity technology; LGALS3BP, galectin 3-binding protein; lyso-PC, lysophosphatidylcholine; MCT, moderate-complexity technology (requires special sampling and processing protocols); MetS, metabolic syndrome; MET, hepatocyte growth factor receptor; miRNA, microRNA; MR, moderate reproducibility (requires laboratory adjustments and depends on analytical sensitivity and limit of detection); MRS, magnetic resonance spectroscopy; MS, mass spectrometry; ncRNA, non-coding RNA; PE, phosphoethanolamine; SERPINC1, serpin C1; SHBG, sex hormone-binding globulin; SNP, single nucleotide polymorphism; T2D, type 2 diabetes.

the assessment of NAFLD and NASH risk and severity (TABLE 6) and their roles in the dynamic assessment of intervention response (TABLE 7).

Genetic markers — specifically DNA sequence variation, including single nucleotide polymorphisms (SNPs) — that are present at birth might be valuable for the identification of individuals at risk of NAFLD or NASH in large screening programmes. To date, three genetic variants have been identified (rs738409, rs58542926 and rs780094), located in *PNPLA3*, *TM6SF2* and *GCKR*, respectively, that have consistently shown an effect on the phenotypic variance of NAFLD and histological outcomes. The risk effect of the common missense SNP rs738409 (minor allele frequency (MAF; the frequency at which the second most common allele occurs in a population) 0.38) is the strongest SNP reported for a genetic modifier of NAFLD. However, it accounts for only ~5.3% of the total variance and a moderate odds ratio (~3.3) of NAFLD and NASH[97]. The low-frequency missense SNP rs58542926 (MAF 0.07) and the intronic SNP rs780094 (MAF 0.30) variants are both associated with a very modest risk (approximately 2-fold[98] and 1.2-fold[99], respectively) of developing NAFLD. It is then not surprising that the diagnostic and prognostic performance of detecting NAFLD and/or discriminating between NAFL and NASH are not improved by the incorporation of genetic variants into the biomarker agenda compared with the incorporation of non-genetic markers[20,100–102] (TABLE 7). In fact, although their inclusion alongside routine clinical parameters results in a reasonable ability to predict the presence of the disease and the probability of progression, accuracy is comparable to that of existing biomarker scores that combine clinical and routine laboratory variables[20,100–102]. Conversely, available evidence indicates that rs738409 might assist in the identification of patients who are more likely to have reduced liver fat levels after lifestyle intervention or bariatric surgery[103,104] (TABLE 7).

Genomic information derived from transcriptomics that includes expression of non-coding RNAs, specifically microRNAs (miRNAs), probably improves robustness in discriminating patients with NAFLD who are at risk of developing advanced disease, as they provide precious information on molecular signatures associated with an aggressive phenotype, for instance, liver inflammation and fibrosis. In addition, non-coding RNAs produced by the affected tissue can be targeted, which is the hallmark of an ideal biomarker for assessing dynamic changes (the course of the disease). Assessment of cell-free RNA or cell-free DNA (cfDNA), which are nucleic acid molecules that circulate in serum or plasma, is particularly noteworthy. In the context of liver diseases, miR-122 has been extensively studied for this liquid biopsy approach and has been shown to be valuable in predicting the presence of NASH[105–107] (TABLE 6). In one study, miR-122 had an AUROC of 0.71 for diagnosing

Table 7 | **Biomarkers for the dynamic assessment of intervention response in NAFLD and NASH**

Study details	Feasibility	Description	Accuracy	Limitations and reproducibility	Refs
SNP (rs738409) in DNA from peripheral blood					
18 patients with NAFLD; liver fat content assessed by MRS	LCT, HR and A	• Patients enrolled in a programme of hypocaloric low-carbohydrate diet • Outcomes: changes in liver fat content, insulin sensitivity, BMI and energy expenditure • Participants stratified into two groups: homozygous GG versus homozygous CC	• Absolute change (% reduction) in liver fat content was influenced by homozygosity status of the risk G allele (for GG versus CC, 3.7% ± 0.5% versus 2.0% ± 0.6%) • There was a lack of influence of rs738409 genotypes on the remaining outcomes	• Pilot study that needs replication and validation; small sample size • Histological changes could not be evaluated	103
154 patients with NAFLD; liver fat content assessed by MRS	LCT, HR and A	• Patients enrolled in a lifestyle modification programme (increased energy expenditure and reduced caloric intake for 12 months) • Outcomes: changes in liver fat content and remission of NAFLD	• Change (% reduction) in liver fat content influenced by the risk G allele (GG: 8.3% ± 8.5%, GC: 3.6% ± 5.5% and CC: 2.8% ± 5.8%) • Remission of NAFLD was not influenced by rs738409 genotype	Histological changes could not be evaluated	125
84 patients who were morbidly obese and had NAFLD	LCT, HR and A	• Patients enrolled in a bariatric surgery programme; NAFLD assessed by liver biopsy (before surgery) and MRS (12 months after surgery) • Outcomes: remission of NAFLD and changes in body weight • Other SNPs assessed (rs58542926 and rs641738)	• Median weight loss (47 kg versus 38 kg) and changes in liver fat content were higher in carriers of the rs738409 G risk allele as compared with C allele carriers • No influence of rs58542926 and rs641738 in any of the assessed outcomes	• Histological changes could not be evaluated • There were non-assessed concomitant environmental factors (low-fat liquid diet before surgery might have influenced the observed effects)	104
Composite biomarker panel					
238 patients with NAFLD (NASH score ≥ 3) enrolled in the GOLDEN-505 trial (elafibranor)	MCT, MR and A	• Combination of laboratory tests (HbA_{1c}), circulating biomarkers (YKL40 and α_2m) and miR-34a • Outcomes: identification of patients at risk of fibrosis progression to be included in pharmacotherapy	AUROC (fibrosis progression) 0.82 (Sn 73%, Sp 78%, PPV 72%, NPV 79%)	Needs cross validation in longitudinal cohorts	108

α_2m, α_2-macroglobulin; A, affordable; AUROC, area under the receiver-operating characteristics curve; HbA_{1c}, haemoglobin A_{1c}; HR, high reproducibility; LCT, low-complexity technology; MCT, moderate-complexity technology (requires special sampling and processing protocols); MR, moderate reproducibility (requires laboratory adjustments and depends on analytical sensitivity and limit of detection); MRS, magnetic resonance spectroscopy; NPV, negative predictive value; PPV, positive predictive value; Sn, sensitivity; SNP, single nucleotide polymorphism; Sp, specificity; YKL40, also known as CH3L1.

NASH and 0.61 for fibrosis[86]. Overall, because miRNAs in the circulating compartment are very stable, their detection in plasma or serum has resulted in a highly reproducible and consistent surrogate of liver injury and damage in patients with different liver diseases[105–108]. Likewise, cfDNA, particularly methylated DNA, has opened new avenues in the biomarker discovery of liver fibrosis[109] (TABLE 6). Nevertheless, many analytical and technical challenges lie ahead — for instance, cfDNA circulates fragmented and at very low concentration, and methylated DNA requires bisulfite treatment — before it can be utilized in clinical contexts.

New omic markers

The development of omics technologies has allowed a hypothesis-free approach to identify novel biomarkers of NAFLD, NASH and fibrosis. To date, findings have mainly been exploratory and cross-sectional with a lack of validation studies. In addition, the complicated methodology involved in omics platforms also prevents widespread application beyond the research setting. Nevertheless, the studies outlined in this section detail a number of promising diagnostic biomarkers for NAFLD and NASH.

Lipidomics is a logical strategy to pursue in NAFLD, with targeted and untargeted approaches identifying substantial alterations in multiple plasma lipid species and eicosanoids across the spectrum of NAFLD[39,40,110,111]. A large study of 679 patients who underwent liver biopsy or MRS discovered and validated a signature comprising three lipid molecules that was modestly accurate (AUROC 0.71–74) at predicting NAFLD[112]. Similar smaller studies have found circulating oxidized fatty acids and products of arachidonic acid metabolism to be predictive of NASH; however, these findings require larger confirmatory validation studies[39,40,111].

Small studies involving proteomic approaches have highlighted increases in serum gamma-glutamyl dipeptides in NASH, which are involved in metabolism of glutathione, a key antioxidant in the liver[113,114]. A large study of 318 patients who underwent liver biopsy used a combination of genetic, clinical, lipidomic and metabolomics approaches to derive the NASH ClinLipMet score, which could identify patients with NASH with an accuracy (AUROC) of 0.86–0.88 and was not affected by statin medications or degree of obesity[101]. The complexity and laboratory expertise required to calculate the score currently limit widespread utilization. Notably, the metabolomics alterations in this study included changes in circulating levels of branch chain and essential amino acids, including glutamate

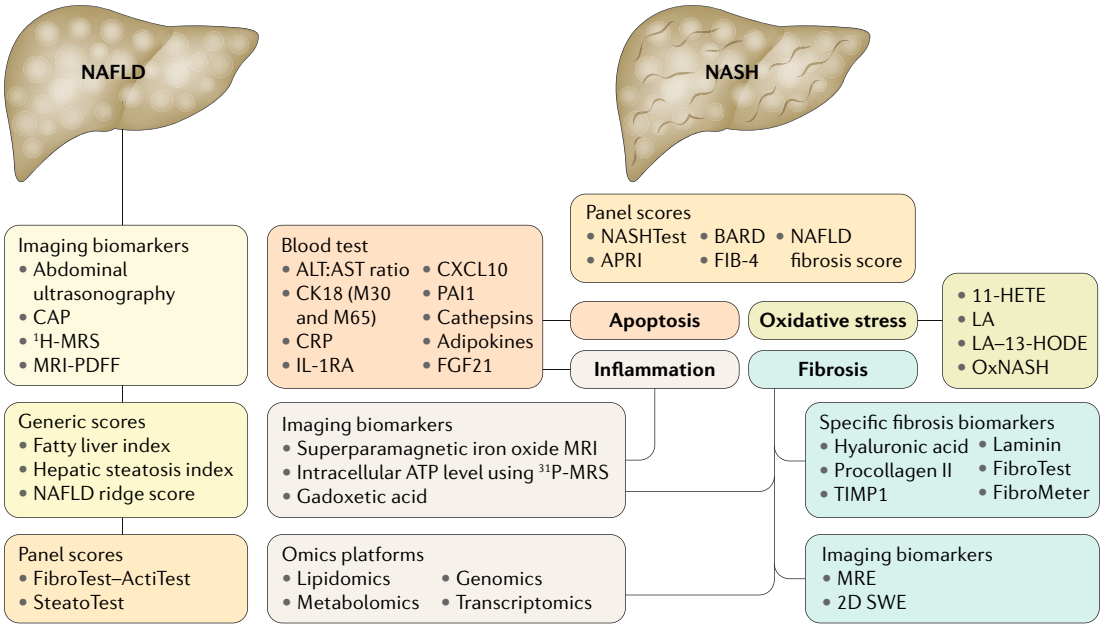

Fig. 3 | **Noninvasive assessment of NAFLD and NASH.** For routine clinical practice, abdominal ultrasonography remains the standard test to detect fatty liver. Other steatosis tests can be used in research or to diagnose fatty liver in patients with milder steatosis. Ultrasound-based and MRI-based measurements of liver stiffness or elasticity are already acceptable tests to detect advanced fibrosis or cirrhosis, whereas simple serum formulae have reasonable negative predictive value in excluding advanced disease. The optimal biomarker for NASH remains elusive, but a number of approaches based on biology and omics platforms are under intensive evaluation. ALT, alanine aminotransferase; APRI, AST:platelet ratio index; AST, aspartate aminotransferase; CAP, controlled attenuation parameter; CK18, cytokeratin 18; CRP, C-reactive protein; CXCL10, CXC-chemokine ligand 10; FGF21, fibroblast growth factor 21; FIB-4, Fibrosis-4 index; ^{1}H-MRS, proton magnetic resonance spectroscopy; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; IL-1RA, IL-1 receptor antagonist protein; LA, linoleic acid; MRE, magnetic resonance elastography; MRI-PDFF, MRI-estimated proton density fat fraction; ^{31}P-MRS, phosphorus magnetic resonance spectroscopy; PAI1, plasminogen activator inhibitor 1; SWE, shear wave elastography; TIMP1, tissue inhibitor of metalloproteinases 1.

and serine. These amino acids had previously been identified as potential biomarkers of NASH by an in silico study using a systems biology approach based on a genome-scale metabolic hepatocyte model[115].

Metabolomics has also been used to differentiate metabolic subtypes of human NAFLD on the basis of metabolite profiles observed in a genetic animal model of NASH with decreased hepatic levels of *S*-adenosylmethionine (SAMe)[116]. The authors suggest that metabolite profiling can identify a subtype of NAFLD that responds to SAMe supplementation, thereby suggesting that a biomarker can be used in the future to stratify treatment approaches.

New omics technology has led to an explosion of interest in the microbiome and the profiling of gut microbiota and metabolites that associate with NAFLD, NASH and fibrosis[117–120]. Although findings have been mixed, alterations in the phyla Firmicutes (reduced) and Proteobacteria (increased) and reduced *Ruminococcus* at both the genus and species level have been associated with NASH and fibrosis in several studies in humans[119,120]. Meta-genomic sequencing of stool microbiota from 86 individuals with NAFLD identified 37 species associated with advanced fibrosis, enabling the development of an algorithm that could predict advanced fibrosis with a high degree of accuracy (AUROC 0.936)[120]. Subsequent identification of serum metabolites predicted by gut bacterial functional analysis found differential levels of 11 amino acids and metabolites involved in nucleoside and carbon metabolism, suggesting that a serum test based on gut microbiome profiles will be a useful marker in the future.

Last, analysis of volatile organic compounds, or 'volatilomics', in stool and breath has demonstrated discrete differences in profiles in patients with NAFLD and NASH[117,121]. These studies have been relatively small (<100 patients), and the accuracy for distinguishing NASH and non-NASH is modest (AUROC 0.77)[121]. Although these studies relied upon mass spectrometry of breath samples, an interesting study published in 2016 utilized a portable gas-sensor array (an 'electronic nose') to determine the breath-print associated with liver cirrhosis among patients with chronic liver disease from a variety of aetiologies, raising the possibility that this technology might be able to distinguish different stages of NAFLD[122].

Conclusions

The past decade has seen major developments in noninvasive assessments for NAFLD and NASH. For routine clinical practice, abdominal ultrasonography remains the primary test to diagnose fatty liver because of its wide availability and relatively low cost (FIG. 3). Serum formulae have limited accuracy for individual patients but can be easily applied in large epidemiological studies. CAP

and liver stiffness measurement by vibration-controlled transient elastography enable simultaneous assessment of liver fat and fibrosis, and the criteria for valid results are well defined[123,124]. This approach can be used as initial assessment of patients at high risk in centres where the equipment is available. However, longitudinal studies are needed to define how well serial measurements reflect disease progression and treatment response. MRI-PDFF and MRE are superior to transient elastography and other noninvasive tests of steatosis and fibrosis and have already been applied in early-phase NASH trials. Cost and availability will be the main hurdles for their wider application.

Compared with noninvasive tests for hepatic steatosis and fibrosis, the development of NASH biomarkers has lagged behind, in part owing to the complex biology and dynamic activity of NASH. Nonetheless, with obeticholic acid, elafibranor, selonsertib and cenicriviroc entering phase III development, the development of a noninvasive NASH biomarker to select patients for treatment and monitor treatment response will be a pressing need. Obviously, when the drugs are used in the wider community, it is unrealistic to perform serial liver biopsies on all treated patients. Fortunately, these large clinical trials with long-term follow-up offer a unique opportunity for biomarker discovery and validation. Genetic studies and an omics approach have also shed light on the pathophysiology of NASH and have provided a number of potential biomarkers for further evaluation.

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Author contributions
All authors researched data and contributed to writing and reviewing the manuscript.

Competing interests
V.W.-S.W. served as a consultant for AbbVie, Allergan, Gilead Sciences, Janssen, Perspectum Diagnostics and Pfizer and a speaker for Bristol-Myers Squibb, Echosens, Gilead Sciences and Merck. L.A.A. has received speaking fees from Bayer and holds patents for Hepascore (Quest Diagnostics) for which his employer (University of Western Australia) has received royalties from Quest Diagnostics for its commercialization. V.d.L. has served as a consultant for AbbVie, Bristol-Myers Squibb, Echosens, Gilead Sciences, Merck, Intercept Pharma and Supersonic Imagine and a speaker for AbbVie, Bristol-Myers Squibb, Echosens, Gilead Sciences, Intercept Pharma and Merck. G.L.-H.W. has served as an advisory committee member for Gilead Sciences and a speaker for Abbott, AbbVie, Bristol-Myers Squibb, Echosens, Furui, Gilead Sciences, Janssen and Roche. S.S. declares no competing interests.

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