NonInvasive Biomarkers in Nonalcoholic Fatty Liver Disease: Are We There Yet?

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Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. NAFLD encompasses a spectrum of disease ranging from simple steatosis (NAFL) to nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma. However, despite the growing recognition of this important disease burden, there are significant challenges to accurately and noninvasively diagnose the various forms of NAFLD, especially to differentiate benign steatosis from the progressive NASH. This is of utmost importance because although liver biopsy is considered the current imperfect 'gold' standard for diagnosing NASH and staging fibrosis, it is an invasive procedure with significant limitations. Although, a number of noninvasive markers have been or are currently undergoing investigation, until date, no highly sensitive and specific tests are available to differentiate NASH from simple steatosis. At the moment, further investigations are needed before prediction models or blood-based biomarkers become available and acceptable for routine clinical care. There is a great need for developing inexpensive, easily accessible, highly sensitive and specific biomarkers that permit not only the identification of patients at high risk of adverse outcomes, but also the monitoring of disease progression and response after therapeutic interventions. (J Clin Exp Hepatol 2020;10:88–98)

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, with a global prevalence of 25.24%.[1,2] NAFLD encompasses a spectrum of diseases ranging from simple steatosis (NAFL) to nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma.[3] Today, NAFLD is the second leading cause for liver transplantation in the United States.[4] It is important to distinguish between NASH and NAFL as NASH is the current target for pharmacotherapy. Routine radiologic techniques can detect NAFLD but cannot satisfactorily diagnose NASH or liver fibrosis. Thus, liver biopsy is still the gold standard for diagnosing NASH and differentiating between the different categories of NAFLD. However, liver biopsy is invasive and expensive and has low patient acceptance. It is neither suitable for diagnosis and disease monitoring in clinical settings nor for population screening.[5] Besides, liver biopsy also has disadvantages such as sampling error and intraobserver and inter-observer variability.[6] Hence, noninvasive tests, such as serum biomarkers, are badly needed for screening of NAFLD, differentiation between the different entities of the NAFLD spectrum and prognostication and use in management and therapeutic clinical trials. Thus, there is a great opportunity for noninvasive biomarkers in the various fields of NAFLD including diagnosing NASH, and assessing fibrosis.

There have been considerable advances in the development and validation of noninvasive biomarkers of NAFLD in the last decade. There are two targets for application of noninvasive biomarkers in NAFLD. The first is differentiation of steatohepatitis from simple steatosis; this is important as the prognosis of NASH is different from simple steatosis.[7] The second is the identification of fibrosis because this is the most important determinant of progression/regression, prognosis and treatment decisions.[8] Many markers of inflammation, hepatocyte apoptosis, fibrosis and oxidative stress have been investigated for diagnosing inflammation and fibrosis in NAFLD. This article briefly reviews the current status of available noninvasive biomarkers for identifying NASH and advanced fibrosis in patients with NAFLD.

BIOMARKERS FOR PREDICTION OF NASH

There are many biomarkers that have been investigated to predict NASH in patients with NAFLD. These range from clinical (age, gender, diabetes, body mass index [BMI]), biochemical (aminotransferases, bilirubin and ferritin), metabolic (glycated haemoglobin, insulin and homeostatic model assessment of insulin resistance [HOMA-IR]) and lipid (triglycerides, cholesterol) parameters to markers that reflect specific and complex molecular mechanisms underlying the pathogenesis and progression of NAFLD, including inflammation, oxidative stress, apoptosis and glucose and lipid metabolism. A single biomarker is

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unlikely to discriminate between simple steatosis and NASH as the pathogenesis of NASH is complex, and it involves multiple biological aberrations. Thus, most of the current models include multiple variables to add robustness to the noninvasive prediction models.

Clinical and Biochemical Models

Liver enzymes such as alanine aminotransferase (ALT) or aspartate aminotransferase (AST) are associated with some histological features such as inflammation and steatosis. However, correlations appear to be influenced by disease severity.[9] Normal AST and ALT do not rule out NASH. Liver enzymes do not correlate with the degree of fibrosis.[10] Clinical models using clinical features such as presence of diabetes, hypertension, BMI, sleep apnoea, age and gender in combination with laboratory parameters have been extensively investigated and correlated with NASH. However, they are erroneous when used alone.[11–14] These models perform reasonably well in predicting NASH, with an area under the receiver operating characteristic curve (AUROC) of 0.76–0.80, and some of them yield acceptable negative predictive values (NPVs) of 80–93%, when using their lower cut-offs to exclude patients with NASH.[11–14]

Markers of Inflammation

Patients with NASH have increased levels of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Wieckowska *et al*[15] showed that there is strong association between IL-6 and NASH. However, IL-6 is raised in several other inflammatory conditions and triggers fibrosis in unresolved inflammation. IL-6 has anti-inflammatory activity and is also involved in the regulation of metabolic, regenerative and neural processes.[16] The TNF-α level is increased severalfold in NASH; however, it is also increased in several inflammatory diseases, cancer and infections. Ajmera *et al*[17] evaluated plasma biomarkers in 648 patients with biopsy-proven NAFLD and showed that increased activated plasminogen activator inhibitor-1 (PAI-1) had a strong association with definite NASH. This finding confirms the previous observations by Verrijken *et al*.[18] PAI-1 is a serine protease inhibitor and primary regulator of the fibrinolytic system. Interestingly, because of its procoagulant properties, PAI-1 has been hypothesised as a potential link between NAFLD and its associated cardiovascular risk.[18]

Markers of Cell Death

Markers of apoptosis/cell death have been used to differentiate simple steatosis from NASH.[19] Activation of caspase 3 results in cleavage of cytokeratin 18 (CK18), which is a major intermediate filament protein in hepatocytes, and CK18 can be measured in serum by immunoassay.[20] Serum CK18-M30 fragment concentrations correlate well with severity of NASH.[21] Two recent meta-analyses have reported a pooled AUROC of 0.82 (0.76–0.88) for discriminating patients with NASH with a median sensitivity and specificity of 66–78% and 82–87%, respectively.[20,22] A two-step approach using CK18 and FGF21 may further improve accuracy in diagnosing NASH.[23] Levels of CK18-M65 have also been used as biomarkers of total cell death, and in a study, it had a similar AUROC to that of CK18-M30 (0.82) in detecting NASH.[24] Besides, changes in CK18-M65 also correlate with histological progression.[25] Other combinatorial models that add CK18 to soluble Fas,[26] adipocytokines,[24] clinical (diabetes, gender, BMI) and routine blood-based parameters (ALT, platelets and triglycerides) showed better NASH prediction in patients with NAFLD.[27,28] A model combining CK18 fragments with the C-terminal cleavage site of procollagen type-III N-terminal peptide (Pro-C3), acetyl-high mobility group box 1 and patatin-like phospholipase domain–containing protein 3 (PNPLA3) rs738409 significantly improves the accuracy of NASH diagnosis (AUROC = 0.87, sensitivity: 71% and specificity: 87%) in patients with NAFLD.[29] However, this needs further validation.

Markers of Oxidative Stress

Markers of oxidative stress may be useful as biomarkers of disease. However, these substances are relatively volatile and not always easily measured in serum. The relative importance of mitochondrial, peroxisomal, CYP450, nitric oxygen synthetase and myeloperoxidase pathways is not yet clear.[30] Products of free radical–mediated oxidation of linoleic acid (9- and 13-hydroxyoctadecadienoic acid and 9-13-oxo-octadecadienoic acid) measured in plasma were significantly elevated in patients with NASH with reference to patients with fatty liver without inflammation or patients with normal biopsies.[31] Malondialdehyde, thiobarbituric acid reactive substances and oxidised low-density lipoprotein have all been assessed as markers of oxidative stress in patients with NASH but with conflicting results.[32,33] The interaction of molecules such as oxidised low-density lipoprotein and thiobarbituric acid reactive substances with stellate cells may be important in promoting fibrosis.[34]

Adipocytokines

Adipocytokines are involved in the pathogenesis of NAFLD and have been strongly related to its severity. There is an association between circulating levels of adiponectin, leptin, ghrelin, IL-6 and TNF-α and insulin resistance, diabetes, obesity and dyslipidaemia, which may explain their potential role in the progression of NAFLD. Machado *et al*[35] found that a panel including adiponectin, leptin and ghrelin could differentiate patients with NASH and simple steatosis with an AUROC of 0.79.

Predictive Models to Distinguish NASH from Simple Steatosis

A number of predictive models have been developed and validated to differentiate simple steatosis from NASH. These include the HAIR (hypertension, ALT, insulin resistance) score, which gives an AUROC of 0.9,[36] and the NashTest® (consisting of 13 variables including age, gender, weight, height, triglycerides, cholesterol, total bilirubin, ALT and AST, gamma-glutamyl transferase [GGT], fasting blood glucose, α2-macroglobulin, haptoglobin and apolipoprotein A), which has an AUROC of 0.79, for differentiation of NASH from simple steatosis.[37] The 'FibroTest' (marketed as 'FibroSure' in the United States) is a hepatic damage score that is useful in a variety of diseases involving the liver. It is derived from age, gender and five serum markers.[38] The markers are α2-macroglobulin, haptoglobin, apolipoprotein α1 (APOA1), GGT and total serum bilirubin. ALT has also been used in another subtest called ActiTest, for measuring necroinflammatory activity in patients with chronic hepatitis B and C. The 'SteatoTest' combines α2-macroglobulin, haptoglobin, APOA1, total bilirubin, GGT, fasting glucose, triglycerides, cholesterol and ALT, parameters adjusted for the patient's age, gender, weight and height.[38] The Fibromax® panel is the combination of the FibroTest, SteatoTest and NashTest, and it improves diagnostic accuracy further.[39] Campos *et al*[11] described a NASH clinical scoring system using AST, hypertension, presence of type 2 diabetes, ALT, obstructive sleep apnoea and nonblack ethnicity. This system has an AUROC of 0.75 for diagnosis of NASH.[11] NASH diagnostics using a combination of CK18-M30 and CK18-M65 levels with adiponectin and resistin values yielded an AUROC of 0.91 in the test and 0.73 in the validation groups. A recent meta-analysis has evaluated the performance of the NashTest® and ActiTest® for the diagnosis of NASH in 494 patients with obesity with a prevalence of NASH of 17.2%. The weighted AUROC was significant for the diagnosis of NASH at 0.84 (0.82–0.86, $P < 0.0001$).[40]

BIOMARKERS FOR PREDICTION OF FIBROSIS

Fibrosis stage is a major determinant of all-cause and liver-related mortality in patients with NASH.[41,42] Therefore, noninvasive assessment of fibrosis severity is crucial in the management of patients with NAFLD. The importance of staging the disease in the context of fibrosis across liver diseases in general is thus manifold. First, in the development of treatment decision algorithms, this is particularly relevant in adult viral hepatitis. Second, functional tests may be even better than biopsy or measurement of the hepatic vein pressure gradient in predicting the outcome and thus planning appropriate follow-up and intervention.[13,43] Finally, the diagnosis of cirrhosis is important because surveillance for varices and hepatocellular carcinoma may be instituted. These issues are uniformly applicable across the spectrum of chronic liver diseases, not NAFLD alone.[44,45]

Demographics and Simple Blood Tests

Noninvasive markers of fibrosis include simple bedside tests and indices that have been studied in large cohorts of patients with liver disease. Ferritin an intracellular protein present in all cells, binds to iron and releases it in a controlled fashion. The ferritin level increases in response to infection and inflammation. Serum ferritin is an independent predictor of advanced hepatic fibrosis among patients with NAFLD.[46] The indices include the AST:platelet ratio index,[47] the AST:ALT ratio,[48] FIB-4[49] and Forn's index.[47] These tools have been validated in the NAFLD population with an AUROC between 0.67 and 0.86 for differentiation of severity of fibrosis.[50,51] The BAAT score (consisting of BMI, ALT, age and triglyceride levels) has an AUROC of 0.86 for prediction of no fibrosis, 0.75 for F2, 0.92 for F3 and 0.81 for cirrhosis in NAFLD.[52] The BARD (BMI, AST/ALT ratio, diabetes) score was developed in a cohort of 827 patients with NAFLD and was found to be useful in excluding patients without advanced NAFLD with an AUROC of 0.81.[53,54] The NAFLD fibrosis score (NFS) (incorporating presence of diabetes, AST, ALT, BMI, platelets and serum albumin) yields an AUROC of 0.88 for advanced fibrosis.[55] This was validated by Shah *et al*[50] with an AUROC of 0.77 for advanced fibrosis and by McPherson *et al*[56] with an AUROC of 0.84. The FIB-4 index was validated by Shah *et al*[50] for advanced fibrosis in patients with NAFLD, with an AUROC of 0.802.[50] McPherson *et al*[56] also found the FIB-4 score provides the best diagnostic accuracy for advanced fibrosis, with an AUROC of 0.86.[56] These noninvasive scoring systems, including the NFS, FIB-4 index, AST:platelet ratio index and BARD score, yield high NPVs but poor positive predictive values (PPVs), suggesting that they are best applied to exclude subjects without advanced fibrosis, thereby avoiding unnecessary liver biopsies.

A meta-analysis compared the diagnostic efficacy of the NFS, FIB-4 index and BARD score for detecting advanced fibrosis. The pooled AUROC was 0.84 and 0.85 for NFS (cut-off = −1.455) and FIB-4 (cut-off = 1.30), respectively, when their low cut-offs were used to rule out advanced fibrosis. But when high thresholds were used to diagnose advanced fibrosis, the pooled AUROC was 0.65 and 0.84 for NFS (cut-off = 0.676) and FIB-4 (cut-off = 3.25), respectively. A BARD score of 2 provided lower diagnostic accuracy than the NFS and FIB-4 score (AUROC = 0.76).[57] These results indicate that both FIB-4 thresholds (1.30 and 3.25) have good diagnostic accuracy to discriminate patients with advanced fibrosis, whereas a cut-off of −1.455 for the NFS may accurately exclude patients with advanced fibrosis. Despite the NFS and FIB-4

displaying good diagnostic efficacy, many patients (30%) fall in between the lower and upper threshold values (indeterminate results), and many factors such as age, diabetes and prevalence of fibrosis, among others, may influence their diagnostic performance. Recently, new age-adjusted cut-offs have been proposed to improve the diagnostic efficacy of the NFS and FIB-4 for advanced fibrosis.[58]

Biomarkers of Extracellular Matrix Turnover

These biomarkers measure the degree of extracellular matrix (ECM) turnover. ECM markers are a more direct method of assessing fibrogenic activity and will tend to measure a dynamic process rather than a static one. Hyaluronic acid (HA) that is synthesised by stellate cells and metabolised by sinusoidal endothelial cells is one of the most validated markers of fibrosis in liver disease.[59,60] HA has been found to be an accurate marker of fibrosis in NAFLD.[61,62]

The FibroTest has shown good predictive values for diagnosing advanced fibrosis (AUROC = 0.88) in patients with NAFLD. However, its diagnostic performance may be reduced in the presence of acute inflammation, sepsis and extrahepatic cholestasis.[63]

Hepascore is used to detect significant fibrosis in many chronic liver diseases. It combines clinical variables of age and gender with five blood-based parameters including bilirubin, gamma-glutamyl transferase, HA, and α2-macroglobulin.[64] In patients with NAFLD, a threshold of 0.37 helps to identify individuals with advanced fibrosis, with an AUROC of 0.81 and NPV and PPV of 97% and 60%, respectively.[65]

Fibrometer™ incorporating age, weight, fasting glucose, AST, ALT, ferritin and platelets has also been validated for prediction of fibrosis in an NAFLD population.[51] The test demonstrated an AUROC of 0.94 for significant fibrosis, 0.9 for severe fibrosis and 0.9 for cirrhosis.

The enhanced liver fibrosis (ELF) test is another test that includes HA, procollagen type III N-terminal peptide (PIIINP) and tissue inhibitor of metalloproteinase 1 (TIMP-1). This has consistently demonstrated good predictive values for identifying patients with advanced fibrosis, with an AUROC of 0.90, sensitivity of 80% and specificity of 90%, using a cut-off of 10.35.[66] However, its performance has been found to be considerably influenced by age and gender in patients with chronic hepatitis C (CHC).[67]

PIIINP is a serum marker of collagen turnover, and increased levels occur as a consequence of tissue repair and fibrosis. PIIINP as a single biomarker has been associated with advanced fibrosis in patients with NAFLD. For diagnosing advanced fibrosis, a cut-off of 6.6 ng/ml and 11 ng/ml yielded an NPV and PPV of 95% and 100%, respectively.[68]

Pro-C3 is a new serum marker derived exclusively from collagen III synthesis, and deposition has recently been validated as a predictor of fibrosis progression in patients with CHC.[69] A recent study in 150 patients with biopsy-proven NAFLD reported that Pro-C3 was able to identify patients with advanced fibrosis, yielding an AUROC of 0.91 and an NPV and PPV of 97% and 56%, respectively.[70] Pro-C3 correctly classified 82% of patients using a previously published cut-off (>1.6738).[70] Recently, serum Pro-C3 combined with clinical variables (age, BMI, diabetes and platelets) was found to be superior to established serological fibrosis tests in identifying individuals with NAFLD and advanced fibrosis, yielding an AUROC of 0.87 and 0.85 in derivation and validation cohorts, respectively.[71]

Another model incorporating serum HA, CK18 and TIMP-1 yielded an AUROC of 0.90, with a sensitivity of 88% and specificity of 84% for predicting advanced fibrosis in patients with NAFLD.[72]

FIBROSpect is a test marketed to detect fibrosis in patients with chronic liver disease.[73] This model incorporates α2-macroglobulin, HA and TIMP-1. Data from a recent study involving a large cohort of histologically confirmed patients with NAFLD showed that this panel accurately detects patients with advanced fibrosis with an AUROC of 0.87 and 0.85 in derivation and validation cohorts, respectively.[74]

The various biomarkers used for diagnosis of NAFLD and NASH have been compared in Tables 1 and 2.

NON–HYPOTHESIS-DRIVEN SEARCH FOR NOVEL BIOMARKERS USING NEW TECHNOLOGIES

The use of relatively new, high-throughput techniques such as proteomics, glycomics and microarray in the derivation of panels of biomarkers associated with a disease may also give an insight into the pathophysiology of the condition.

Proteomics

Proteomic technologies have been used successfully for biomarker discovery, generating lists of many candidate protein biomarkers to identify NASH and advanced fibrosis in patients with NAFLD. Ladaru *et al*[75] performed a systematic review and summarised 22 studies; of which, twenty-one confirmed serum protein biomarkers. Unfortunately, none of these candidate proteins have been translated into commercially available diagnostic tests. Bell *et al*[76] conducted a serum proteomics and biomarker discovery study in 69 patients with well-characterised NAFLD (simple steatosis: 24, steatohepatitis without advanced fibrosis: 23, steatohepatitis with stage 3/4 fibrosis [F3/4]: 22) and 16 controls without NAFLD. Using a label-free mass spectrometry–based approach, these investigators

Table 1 Panels of Serum Biomarkers for Fibrosis in NAFLD.

Biomarker panel	Components	Study subjects	Results (AUROC)	Diagnostic accuracy	References
	AST/ALT	174 patients with NAFLD	0.83 in the training set 0.83–0.9 in the validation set	Cut-off = 0.8, NPV: 93%, PPV: 44%	57
	Pro-C3 levels	150 patients with NAFLD	0.91	Cut-off >1.6738 82% correctly classified	71
	Terminal peptide of procollagen III	136 patients with NAFLD	0.82 in the training set 0.84 in the validation set	Cut-off = 6.6 ng/ml, NPV: 95% Cut-off = 11 ng/ml, PPV: 100%	69
APRI score	AST, platelets	541 patients with NAFLD	0.73	Cut-off = 1, NPV: 84%, PPV: 37%	57
FibroTest/FibroSure	Haptoglobin, α2-macroglobulin, apolipoprotein A, bilirubin and GGT	1202 patients with NAFLD	0.86 in the training set 0.85 in the validation set	Cut-off = 0.3, NPV: 98% Cut-off = 0.7, PPV: 60%	63,64
ELF test	Hyaluronic acid, PIIINP and TIMP-1	1329 patients with NAFLD	0.87 in the training set 0.90 in the validation set		67
NAFLD fibrosis score	Age, BMI, albumin, AST/ALT ratio, hyperglycaemia and platelets	733 patients with NAFLD	0.88 in the training set 0.77–0.84 in the validation set	Cut-off = 0.81, NPV: 78–93% Cut-off = 0.67, PPV: 82–90%	50,56,57,59
Fibrometer	Age, weight, glucose, AST, ALT, ferritin and platelets	235 patients with NAFLD	0.94 in the training set 0.94 in the validation set		52
FIB-4 index	Age, AST, ALT and platelets	686 patients with NAFLD	0.80 in the training set 0.86 in the validation set	Cut-off = 1.30, NPV: 90–95% Cut-off = 2.67, PPV: 80%	50,57,59
BARD score	BMI, diabetes and AST/ALT ratio	1513 patients with NAFLD	0.81 in the training set 0.77–0.78 in the validation set	Cut-off = 2, PPV: 27%, NPV: 95–97%	54,57,59
Hepascore	Age, sex, bilirubin, GGT, α2-macroglobulin and hyaluronic acid	242 patients with NAFLD	0.81	Cut-off = 0.37, PPV: 57%, NPV: 92%	65,66
FIB-C3	Pro-C3, age, BMI, diabetes and platelets	433 patients with NAFLD	0.86 in the training set 0.85 in the validation set		72
FIBROSpect test	a2-macroglubulin, hyaluronic acid and TIMP-1	792 patients with NAFLD	0.87 in the training set 0.85 in the validation set	Sensitivity: 84–81%, specificity: 72–74%.	75
Novel combinations	HA, CK18 and TIMP-1	180 patients with NAFLD	0.90	Sensitivity: 88.2%, specificity: 84.1%	73

ELF, enhanced liver fibrosis; APRI, AST/platelet ratio index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under the receiver operating characteristic curve; BMI, body mass index; CK, cytokeratin; INR, international normalised ratio; GGT, gamma-glutamyl transferase; HA, hyaluronic acid; NAFLD, nonalcoholic fatty liver disease; PPV, positive predictive value; NPV, negative predictive value; Pro-C3, C-terminal cleavage site of N-terminal type III collagen propeptide; PIIINP, N-terminal type III collagen propeptide; TIMP-1, tissue inhibitor of metalloproteinase-1.

Table 2 Summary of Biomarkers for Diagnosis of NASH.

Noninvasive test	Parameters	Study subjects	Result (AUROC)	Diagnostic accuracy	References
	Age, gender, AST, BMI, HA, AST:ALT ratio	80 patients with NAFLD; 39 patients with simple steatosis, 41 patients with NASH	0.76	Sensitivity: 74%, specificity: 66%	[54,12]
	Hypertension, DM, AST > 27, ALT > 27, sleep apnoea, nonblack race	200 patients undergoing bariatric surgery: 64 patients had NASH.	0.8	≥6 PPV: 87–93% ≤2 NPV: 93–80%	[51,11]
	DM, TG > 150 mg/dl, ALT, obstructive sleep apnoea	253 patients with morbid obesity	0.76	≤1 NPV: 90% ≥4 PPV: 60%	[17,14]
	Resistin, cleaved CK18, adiponectin	101 patients with NAFLD	0.91 in the test group, 0.73 in the validation set	Cut-off = 0.4320 Sensitivity: 72%, specificity: 91%	[23,24]
	CK18, ALT, platelets, triglycerides	95 patients with NAFLD	0.92	Cut-off = 0.361 Sensitivity: 89%, specificity: 86%	[24,27]
	Adiponectin, leptin, ghrelin	82 patients with morbid obesity	0.79	Sensitivity: 82%, specificity: 76%	[27,35]
	Terminal peptide of procollagen III	136 patients with NAFLD	0.83 in the test group, 0.78 in the validation set	Cut-off = 6.6, NPV: 85–100% Cut-off = 11.0, PPV: 80–100%	[47,69]
	CK18, soluble Fas	177 patients with NAFLD	0.93 in the test group, 0.79 in the validation set	Sensitivity: 88%, specificity: 89%	[22,26]
	CK18 fragments	139 patients with NAFLD	0.83		[19,21]
	CK18 fragment (CK18-M30, CK18-M65, CK18-M65ED) pooled analysis	2415 patients with NAFLD	0.82	CK18-M30 Sensitivity: 68%, specificity: 74%	[20,22]
NashTest	13 variables: age, sex, weight, height, TG, cholesterol, α2-macroglobulin, ApoA1, AST, ALT, haptoglobin, GGT, BR	257 patients (17% patients with NASH) and 383 controls	0.79	Specificity: 94% (PPV = 66%), and sensitivity: 33% (NPV = 81%)	[56,13]
NASH Diagnostics	CK18-M65, CK18-M30, resistin and adiponectin	101 patients with NAFLD, 69 patients in the test group, (32% of patients with NASH) 32 patients in the validation group	AUROC: 0.91	Sensitivity: 96%, specificity: 70%	[43,24]
NAFIC score	Ferritin, fasting insulin, type IV collagen S	619 patients with NAFLD; 177 patients in the test group (95 patients with NASH), 442 patients in the validation group	0.85 in the test group, 0.78 in the validation group		[57,44]
Nice model	CK18-M30, ALT, presence of metabolic syndrome	454 patients obese, 310 patients in the test group, 154 patients in the validation group	0.88 in the test group, 0.83 in the validation set		[58,45]

(*Continued on next page*)

Table 2 (*Continued*)

Noninvasive test	Parameters	Study subjects	Result (AUROC)	Diagnostic accuracy	References
HAIR	Insulin resistance, hypertension and ALT	105 patients with obesity undergoing bariatric surgery, including 26 with NASH		Sensitivity: 80%, specificity: 89%	[48,36]
Novel combinations	Collagen Pro-C3, CK18 (M30) fragments, AST, ALT, procollagen III N-terminal peptide, acetyl-HMGB-1 and PNPLA3 rs738409	374 patients with NAFLD	0.87	Sensitivity: 71%, specificity: 87%	[26,29]

HAIR, hypertension, ALT, insulin resistance; PPV, positive predictive value; NPV, negative predictive value; GGT, gamma-glutamyl transferase; AUROC, area under the receiver operating characteristic curve; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; BR, bilirubin; CK, cytokeratin; DM, diabetes mellitus; HA, hyaluronic acid; NASH, nonalcoholic steatohepatitis; NAFLD, nonalcoholic fatty liver disease; PNPLA, patatin-like phospholipase domain–containing protein A; TG, triglyceride.

identified more than 1700 serum proteins with a peptide ID confidence level of >75%; of which, 605 changed significantly between any two patient groups (false discovery rate <5%). Importantly, expression levels of 55 and 15 proteins changed significantly between the simple steatosis and NASH F3/F4 group and the NASH and NASH F3/F4 group, respectively. The priority 1 proteins with >30% change between any two groups were subsequently considered for developing biomarker candidates. A panel consisting of six priority 1 proteins (fibrinogen β chain, retinol-binding protein 4, serum amyloid P component, lumican, transgelin 2 and CD5 antigen-like) differentiated all four patient groups, with an overall success rate of 76% (AUROC for the control group = 1.0, simple steatosis = 0.83, NASH = 0.86 and NASH with F3/F4 = 0.91). A group of three priority 1 proteins (complement component C7, insulin-like growth factor acid-labile subunit and transgelin 2) correctly categorised 90% of patients as having NAFLD (simple steatosis and NASH) or NASH F3/F4 (AUROC = 0.91). Interestingly, two proteins (prothrombin fragment and paraoxonase 1) were able to totally differentiate control subjects from those with all forms of NAFLD, with an AUROC of 1.0. This group has also reported the results of serum proteomic analysis from their Ossabaw NASH model.[66] This study identified seven priority 1 proteins that were different between pigs which developed NASH and pigs without NASH. Importantly, these investigators found seven priority 1 proteins (apolipoprotein C-III, apolipoprotein β, serum amyloid P component, transthyretin, paraoxonase, protein similar to α2-macroglobulin precursor and orosomucoid I) to be common between their human and Ossabaw swine proteomic investigations.[76,77]

Recently, a new sensitive and quantitative proteomic technology (SOMAscan assay; SomaLogic, Boulder, CO) has been developed. It uses Slow Off-Rate Modified Aptamers (SOMAmers), single-stranded aptamers with modified nucleotides that have a high affinity for specific protein targets in serum, which are subsequently quantified as DNA. In a preliminary study, a proteomic classifier based on eight proteins had an AUROC of 0.932 for identifying steatosis in individuals with obesity undergoing bariatric surgery.[78] Interestingly, a proteomic classifier has performed as well as a multicomponent classifier based on PNPLA3 genotype, proteomics and phenomics at identifying steatosis in this cohort. This approach is now being applied to differentiate various histological stages in patients with biopsy-proven NASH.

Glycomics

Glycosylation is the post-translational modification of secreted proteins, with carbohydrate moieties conveying structural diversity and with a possible role in protein folding and in cell-to-cell interaction including migration, solubility and receptor attachment.[79,80] Changes in glycosylation serve as a good marker of liver dysfunction for a number of reasons. Most glycoproteins in serum (aside from IgG) are synthesized in the liver. Thus, the N-glycome profile will reflect any changes in either the liver or β-cell function. In addition, both the asialoglycoprotein receptor and the mannose/O-linked beta-N-acetylglucosamine receptor in the liver are important in clearing aberrantly glycosylated proteins from the serum. In the presence of architectural disarray, these receptors are decreased in number, and thus, there is a buildup of glycoproteins in serum.[81] With a systems biology approach to the analysis using high-throughput technology, serum N-glycomics may prove to be valuable biomarkers of disease. Previously reported glycomic analysis of liver disease includes the development of the GlycoCirrhotest,[82] the GlycoFibrotest[83] and the GlycoHCC test[84] that can predict the presence of cirrhosis, fibrosis and hepatocellular carcinoma,

respectively, owing to difference in N-glycome patterns. Two recent studies have also investigated the potential of glycomics in noninvasive evaluation of NAFLD.[85–87]

MicroRNAs as Biomarkers in NAFLD

MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression post-transcriptionally. Although miRNAs have been recently implicated in NAFLD, the available data are not robust enough to support their diagnostic use as markers of steatosis, inflammation or fibrosis. miR-122 and miR-34a levels positively correlated with disease severity from simple steatosis to steatohepatitis. In both patients with CHC and patients with NAFLD, serum levels of miR-122 and miR-34a correlated with serum lipids, liver enzymes levels, fibrosis stage and inflammation activity.[88] In a recent study, serum levels of circulating miRNAs, miR-21, miR-34a, miR-122 and miR-451, were found to be associated with NAFLD, and the serum level of miR-122 was correlated with the severity of liver steatosis.[89] Overexpressed microRNA-27α and microRNA-27β influence fat accumulation and cell proliferation during rat hepatic stellate cell activation, but corresponding data from human studies are not presently available or corroborative.[90] In another murine study, Venugopal *et al*[91] reported that liver fibrosis is associated with a downregulation of miRNA-150 and miRNA-194 in hepatic stellate cells and their overexpression causes decreased stellate cell activation. In another study by Alisi *et al*,[92] the miRNA analysis showed significant downregulation of three miRNAs (miR-122, miR-451 and miR-27) and upregulation of three miRNAs (miR-200α, miR-200β and miR-429) in high-fat diet–fed rats (standard diet with high fructose and high-fat diet combined with high fructose).

Gut Microbiota

Loomba *et al*[93] recently reported the utility of a metagenomics signature based on the gut microbiome for noninvasively detecting advanced fibrosis in 86 patients with NAFLD (14 with stage 3 or 4 fibrosis). Their gut microbiome composition was characterised using whole-genome shotgun sequencing of the stool DNA. A random forest classifier, consisting of 40 variables including 37 bacterial species, was able to identify patients with F3/4, with an AUROC of 0.936. However, this study lacked a validation cohort.

Other Noninvasive Approaches

Decaris *et al*[94] recently described a novel method to noninvasively describe the hepatic fibrogenesis flux rates in the liver tissue and blood. They have shown that hepatic fibrogenesis flux rates correlate significantly with the degree of liver fibrosis in 24 subjects who underwent diagnostic liver biopsy for suspected NAFLD.[94] The liver collagen fractional synthesis rate (FSR) and plasma lumican (liver ECM protein) FSR were measured based on 2H labelling using tandem mass spectrometry. In this study, patients undergoing liver biopsy drank heavy water ($2H_2O$, 50 ml two to three times daily) for three to five weeks before their biopsy. The key observations from this study were as follows: (a) there was active remodelling of hepatic ECM even in patients with advanced fibrosis; (b) the hepatic collagen FSR correlated significantly with the fibrosis stage and noninvasive fibrosis indices, such as FIB-4 and liver stiffness, by magnetic resonance elastography; (c) the plasma lumican FSR correlated significantly with the hepatic collagen FSR, liver fibrosis by histology, and noninvasive fibrosis markers such as liver stiffness by magnetic resonance elastography and FIB-4. However, it would have been better if this study would have compared the hepatic collagen FSR and lumican FSR with the ELF score. If this method can be validated in follow-up studies, this could serve as a novel approach to test antifibrotic compounds in short-term proof-of-concept studies. There are several ongoing experimental investigations to examine the utility of blood-based molecular markers (signatures derived from circulating cell-free RNA and DNA methylation and cell-free DNA methylation).[95–97] These are encouraging approaches, but they need to be validated externally using independent cohorts.

SUMMARY

NAFLD has evolved from an unrecognised entity to a heterogeneous liver disease with a common phenotype of having hepatic steatosis over the last forty years. It is increasingly clear that subjects with NASH and especially those with significant fibrosis are at greatest risk of mortality and adverse clinical outcomes.

Despite the growing recognition of this important disease burden, there are significant challenges to accurately and noninvasively diagnose the progressive form of NAFLD. Although liver biopsy is considered the current imperfect 'gold' standard for diagnosing NASH and staging fibrosis, it is an invasive procedure with significant variability in assessment of the key features of NASH.

Thus, a number of serum markers and noninvasive predictive algorithms have been or are currently undergoing investigation. Until date, no highly sensitive and specific tests are available to differentiate NASH from simple steatosis. However, diagnostic accuracy can be improved by combining blood biomarkers. The NFS and FIB-4 can accurately exclude patients with advanced fibrosis because of their high NPVs and because they are inexpensive and easy to obtain. The NFS and FIB-4 are useful screening tools to be routinely applied in clinical practice. Other direct markers of fibrosis such as the ELF test, FibroTest and FibroMeter are expensive and are more specific. They have higher PPVs for detecting patients with

advanced fibrosis. Still, more investigations are needed before prediction models and blood-based biomarkers become available and acceptable for routine clinical care. Hence, biologically based, inexpensive, easily accessible, highly sensitive and specific biomarkers that permit not only the identification of patients at high risk of adverse outcomes but also the monitoring of disease progression and therapeutic response after interventions are urgently needed.

CONFLICTS OF INTEREST

The authors have none to declare.

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