

# Biomarkers in nonalcoholic fatty liver disease

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**BACKGROUND:** Nonalcoholic fatty liver disease (NAFLD) is a chronic liver condition characterized by insulin resistance, type 2 diabetes and fat accumulation in the liver that may cause hepatic inflammation and progressive scarring leading to nonalcoholic steatohepatitis (NASH) and irreversible liver damage (cirrhosis). As a result, there has been increased recognition of the need to assess and closely monitor individuals for risk factors of components of NAFLD and NASH, as well as the severity of these conditions using biomarkers.

**AIM:** To review the biomarkers used to diagnose and define the severity of NAFLD and NASH.

**METHODS:** A comprehensive PubMed and Google Scholar literature search was performed using the terms “non-alcoholic fatty liver disease”, “non-alcoholic steatohepatitis”, as well as the name of each biomarker known to be used. Articles indexed between 2004 and 2014 were used. Each author read the publications separately and the results were discussed.

**RESULTS:** Biomarkers offer a potential prognostic or diagnostic indicator for disease manifestation, progression or both. Serum biomarkers, including total cholesterol, triglycerides, insulin resistance and C-peptide, have been used for many years. Emerging biomarkers, such as apolipoprotein A1, apolipoprotein B, leptin, adiponectin, free fatty acids, ghrelin and tumour necrosis factor- $\alpha$ , have been proposed as tools that could provide valuable complementary information to that obtained from traditional biomarkers. Moreover, markers of cell death and mitochondrial dysfunction (cytokeratins) represent powerful predictors of risk. For biomarkers to be clinically useful in accurately diagnosing and treating disorders, age-specific reference intervals that account for differences in sex and ethnic origin are a necessity.

**CONCLUSIONS:** The present review attempts to provide a comprehensive analysis of the emerging risk biomarkers of NAFLD and NASH, and to use the clinical significance and analytical considerations of each biomarker pointing out sentinel features of disease progression.

**Key Words:** *Adipokines; Apoptosis; Cytokeratin; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Noninvasive biomarkers; Toll-like receptor; Th1/Th2; Tumour necrosis factor*

The obesity epidemic has begun to compromise the health of the population by promoting the premature development of the metabolic syndrome (MS), which significantly increases the risk for liver disease early in life. Approximately 30% to 40% of patients with nonalcoholic fatty liver disease (NAFLD) develop nonalcoholic steatohepatitis (NASH). It is estimated that 10% to 30% of patients with NAFLD develop cirrhosis after 10 years, with NAFLD believed to be the most common cause of cryptogenic cirrhosis. A diet rich in saturated fats and refined carbohydrates leads to hyperinsulinemia and fatty liver. Dietary intervention remains the current standard of care for NAFLD and NASH; however, this intervention often fails to control the disease.

## Les biomarqueurs de la stéatose hépatique non alcoolique

**HISTORIQUE :** La stéatose hépatique non alcoolique (SHNA), une maladie hépatique chronique caractérisée par l'insulinorésistance, le diabète de type 2 et l'accumulation de lipides dans le foie, peut être responsable d'une inflammation hépatique et d'une fibrose progressive qui peut entraîner une stéatohépatite non alcoolique (SNA) et une atteinte hépatique irréversible (cirrhose). Par conséquent, on convient de plus en plus de la nécessité d'utiliser des biomarqueurs pour assurer une surveillance étroite des patients. Cette surveillance portera sur les facteurs de risque de divers aspects de la SHNA et de la SNA, ainsi que sur la gravité de ces maladies.

**OBJECTIF :** Analyser les biomarqueurs utilisés pour diagnostiquer et définir la gravité de la SHNA et de la SNA.

**MÉTHODOLOGIE :** Les auteurs ont effectué une analyse bibliographique approfondie des articles indexés dans PubMed et Google Scholar entre 2004 et 2014 au moyen des termes *non-alcoholic fatty liver disease*, *non-alcoholic steatohepatitis* et du nom de chacun des biomarqueurs qu'ils savaient être utilisés. Chaque auteur a lu les publications séparément et ensemble, ils ont discuté des résultats.

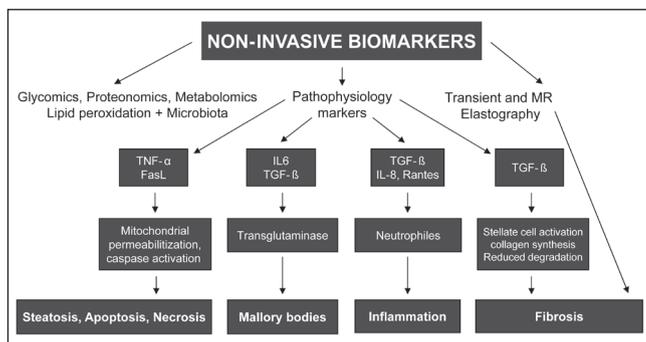
**RÉSULTATS :** Les biomarqueurs procurent un pronostic potentiel d'indicateurs diagnostiques de manifestations ou d'évolution de la maladie, ou de ces deux problèmes. Les biomarqueurs sériques, y compris le cholestérol total, les triglycérides, l'insulinorésistance et le peptide C, sont utilisés depuis de nombreuses années. Des biomarqueurs émergents, tels que l'apolipoprotéine A1, l'apolipoprotéine B, la leptine, l'adiponectine, les acides gras libres, la ghréline et le facteur de nécrose tumorale  $\alpha$ , sont des outils susceptibles de fournir de l'information précieuse, qui complétera celle obtenue grâce aux biomarqueurs habituels. De plus, les marqueurs de mort cellulaire et de dysfonction mitochondriale (les cytokératines) sont de puissants prédicteurs de risque. Pour que les biomarqueurs soient utiles sur le plan clinique pour bien diagnostiquer et traiter ces maladies, il faut obtenir des intervalles de référence propres à l'âge qui tiennent compte des différences en fonction du sexe et de l'origine ethnique.

**CONCLUSIONS :** La présente analyse visait à effectuer un examen approfondi des biomarqueurs émergents du risque de SHNA et de SNA et à utiliser la signification clinique et les considérations analytiques de chaque biomarqueur qui met en lumière les caractéristiques sentinelles d'une évolution pathologique.

NASH, defined as the advanced end of the spectrum of chronic NAFLD, is emerging as an important cause of liver disease. The pathogenesis of NAFLD/NASH and its natural history is captured in liver disease clinics, liver transplantation, diabetes, lipid disorders and obesity. NAFLD/NASH is further studied in pediatric liver and nutrition clinics.

Described by Adler and Schaffner (1) and Ludwig et al (2), NASH is a common manifestation of liver cell injury of various etiologies and of metabolic disorders of fatty acid metabolism. NASH is a chronic liver condition, and can progress to cirrhosis and end-stage liver disease. As the most aggressive form of NAFLD, NASH carries the

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**Figure 1) Noninvasive biomarkers.** Biomarkers can be measured using several technologies including glycomics, proteomics and/or metabolomics, and imaging techniques such as magnetic resonance (MR) and elastography. Pathophysiology: oxidative stress, lipid peroxidation and activity of microbiota can lead to an increase in mitochondrial permeability and caspase activation. At the tissue level, this leads to steatosis, and death by apoptosis and necrosis. An increase in interleukin (IL)-6 and transforming growth factor (TGF)-beta levels leads to transglutamination, which may induce formation of Mallory-Denk bodies in the cells. An increase in TGF-beta, IL8 and RANTES levels result in the recruitment of neutrophils, leading to cellular inflammation. An increase in TGF-beta levels activates stellate cells and leads to collagen secretion as well as a reduction in extracellular matrix degradation. This phenomenon ultimately results in histological fibrosis. FasL Fas ligand; TNF Tumour necrosis factor

highest risk for adverse outcomes (3). Although risk factors for NASH include obesity, insulin resistance and diabetes, the disease can occur in patients of any ethnic origin, sex or body weight (4,5). Body mass index is a known independent predictor of the degree of hepatic fatty infiltration (6). Although the disease predominantly affects the middle-age population, NASH is increasingly recognized in children, commonly in association with obesity (7). The recurrence of NASH in liver-transplant recipients implicates systemic host factors in the development of the disease, rather than liver-related factors (8-11).

Several secondary causes of the typical histological lesions of NASH are described, including total parenteral nutrition; hypobeta-lipoproteinemia; jejuno-ileal bypass; certain forms of bariatric surgery; genetic-metabolic diseases such as hereditary tyrosinemia type 1, galactosemia, Prader-Willi syndrome, glycogen storage disease, lipodystrophy syndromes, sialidosis, fucosidosis, mannosidosis, citrulinemia, argininemia and porphyria cutanea tarda; and malnutrition and acute starvation (12). Natural toxins, environmental toxic substances, as well as several drugs, including amiodarone, didanosine, hydralazine, methotrexate, tamoxifen, perhexiline, coralgil, corticosteroids and calcium channel blockers, can produce macrovesicular steatosis (12). Gut microbiota, producing lipopolysaccharides, may also sensitize the liver to tumour necrosis factor (TNF)-alpha, leading to hepatotoxicity (12). Toll-like receptor-4 causes nuclear factor-kappa B activation and results in TNF-alpha production (12). Histological changes in the liver during NASH are nearly identical to those induced by excessive alcohol intake, as seen in patients with alcoholic steatohepatitis. However, NASH is observed in patients without any evidence of alcohol abuse, which is a flexible definition (13). The separation of NASH from alcoholic liver disease remains based on a history of minimal or no alcohol use. Integral to this definition is the clear identification of a cut-off value for significant alcohol use in light of evidence that even 20 g/day of alcohol can cause hepatic steatosis (14). In addition, underlying host factors may further confer a greater susceptibility for the development of significant liver disease in the setting of chronic alcohol exposure.

It is believed that NAFLD may be the most prevalent form of liver disease in developed countries (15). Fatty liver not associated with alcohol consumption is now recognized as possibly the most common cause

of chronic, asymptomatic liver enzyme elevation in the United States and Europe (16). NASH patients may be asymptomatic or present with mild abdominal pain (17). The liver damage observed in NASH has been well described even though the pathogenesis of the disease remains uncertain. Macrovesicular and/or microvesicular steatosis, ballooning degeneration of hepatocytes, lobular inflammation and, occasionally, cirrhosis characterize the histology of this condition. Steatosis is observed in acinar zone 3, along with zone 3 Mallory bodies and/or acinar zone 3 sinusoidal fibrosis. Morphologically, the mitochondria are swollen, and paracrystalline inclusion bodies can be visualized using electron microscopy. The diagnosis of NASH requires additional morphological evidence of hepatic injury, ranging from inflammation and hepatocellular ballooning to Mallory's hyaline and fibrosis, the latter ranging from minimal to cirrhosis. The histological features of NAFLD/NASH are identical to those of alcoholic liver injury (18).

Biomarkers can be used as unbiased differential indicators of illness onset, aid in the classification of a diseased or nondiseased state, provide the ability to stage disease progression and/or offer insight into its relative severity. An individual's risk of developing a disorder may also be obtained from biomarker research. As such, a prognostic indicator could be used for risk stratification of the general population. In addition to identifying illnesses, the efficacy of clinical or therapeutic interventions aimed toward these disorders may also be obtained.

Figure 1 illustrates a possible strategy of identifying noninvasive biomarkers based on the methodology used and pathophysiology pathway.

#### ABNORMAL LIVER FUNCTION TEST RESULTS

Elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels reflect nonspecific hepatocellular damage. In NAFLD/NASH, aminotransferase levels may be elevated two to four times over the upper limit of normal (19), with ALT being higher than AST, in contrast to alcoholic steatohepatitis. However, in the absence of advanced disease, routine liver function tests are either normal or typically show only mild elevations in aminotransferase levels, with alkaline phosphatase and gamma-glutamyl transferase (GGT) 1.5 to three times the upper limit of normal.

Several studies involving hepatology clinic patients undergoing liver biopsy and morbidly obese individuals undergoing bariatric surgery have found ALT levels to be higher in the presence of NASH than in those with simple steatosis, although this has not been universally observed (19). However, close to 80% of patients with fatty liver in cohort studies have shown ALT levels within normal limits (20). Adams et al (21) reported that aminotransferase levels fall over time as hepatic steatosis and inflammation improve. Aminotransferase levels do not correlate with the degree of fibrosis (22).

The diagnostic accuracy of ALT cut-offs for diagnosing NASH was examined in a series of women undergoing bariatric surgery. Reducing the cut-off from 30 IU/L to 19 IU/L improved sensitivity for the diagnosis of NASH from 42% to 72%. However, this was at the expense of specificity, which fell from 80% to 42% (23). Patients with NAFLD who have high levels of ALT are, therefore, more likely to have inflammation that may lead to NASH.

#### MARKERS OF APOPTOSIS

Cytokeratin (CK)-18 is the major intermediate filament protein of the liver. Caspases cleave CK-18 during hepatocyte apoptosis and create CK-18 fragments that can be detected by immunoassay (24). A recent meta-analysis found a wide range of cut-off values used in M30 assays, based on whether the study authors aimed for 'best sensitivity', 'best specificity' or 'best balance between sensitivity and specificity' to diagnose NASH. Based on the overall analysis, Kwok et al (24) conclude that M30 provides moderate accuracy due to a high variability between cut-offs and respective diagnostic accuracy among studies. Table 1 summarizes recent studies measuring CK-18 and markers of apoptosis (25-36).

Three ELISA-based assays have been recently described to measure CK-18. The M30 assay detects hepatocyte apoptosis through the

**TABLE 1**  
**Biomarkers of apoptosis**

Author (reference); study	Biomarker, matrix, method	Study population, biomarker levels	Associations and diagnostic performance
Fitzpatrick et al (25); 45 pediatric patients with biopsy-proven NAFLD (8 steatosis, 17 borderline NASH and 20 NASH) vs 13 healthy controls	CK-18 in plasma using M30 ELISA	CK-18: median 288 IU/L (IQR 202–494) in NAFLD vs 172 IU/L (IQR 146–205) in controls (P<0.001); CK-18: median 191 IU/L (IQR 167–197) in simple steatosis, 275 IU/L (IQR 191–508) in borderline NASH and 347 IU/L (IQR 258–509) in NASH; CK-18: median 393 IU/L (IQR 225–533) in severe fibrosis vs 243 IU/L (IQR 190–317) in minimal fibrosis (P=0.03)	CK-18: 84% sensitivity, 88% specificity, 90% PPV and 80% NPV as a predictor of NASH at a cut-off of 207 IU/L; CK-18: 83% sensitivity and 40% specificity as a predictor of severe fibrosis at a cut-off of 200 IU/L
Papatheodoridis et al (26); 58 patients with biopsy-proven NAFLD (28 simple steatosis and 30 NASH) vs 40 healthy volunteers	CK-18 in serum using M30 ELISA	CK-18: mean 148 U/L in healthy volunteers vs mean 174 U/L in NAFLD (P=0.013 vs healthy) vs mean 355 U/L in NASH (P<0.001 vs both healthy and NAFLD)	CK-18: sensitivity 70%, specificity 82%, PPV 84%, NPV 73% for diagnosis of NASH at cut-off of $\geq 225$ U/L; CK-18: sensitivity 60%, specificity 93%, PPV 95%, NPV 69% for diagnosis of NASH at cut-off of $\geq 250$ U/L; CK-18: sensitivity 53%, specificity 100%, PPV 100%, NPV 67% for diagnosis of NASH at a cut-off of $\geq 300$ U/L
Lebensztejn et al (27); 52 children with biopsy-proven NAFLD (80.8% overweight, 33 without fibrosis and 19 with fibrosis)	CK-18 in serum using M30 ELISA	CK-18: mean 215 U/L (IQR 150–342); CK-18: mean 311.0 U/L in fibrosis vs 177.5 U/L without fibrosis (P=0.05)	CK-18: 79% sensitivity, 60% specificity, 56% PPV and 82% NPV for fibrosis in NAFLD at a cut-off of 210 U/L
Tamimi et al (28); 95 patients undergoing liver biopsy for suspected NASH (41 NASH and 54 non-NASH)	CK-18 in plasma using M30 ELISA; sFas in plasma using ELISA	CK-18: median 508 U/L (IQR 280–846) in NASH vs median 176 U/L (IQR 131–224) in non-NASH (P<0.001); sFas: median 11.8 ng/mL (IQR 7.8–12.5) in NASH vs median 5.9 (IQR 4.8–8.3) ng/mL in non-NASH (P<0.001)	CK-18 + sFas: 88% sensitivity, 89% specificity, 86% PPV and 91% NPV using a best cut-off point derived from a probability of 36.6% corresponding to a score of $-0.5509$ based on a risk score model of $6.4894 + 0.0078 \times \text{CK-18 fragments (U/L)} + 0.4668 \times \text{sFas (ng/mL)}$
Joka et al (29); 112 patients with chronic liver diseases (22 with NASH/NAFLD based on histological examination) vs 18 healthy individuals	CK-18 in serum using M30 ELISA; CK-18 in serum using M65 ELISA; CK-18 in serum using M65ED ELISA	M30: mean ( $\pm$ SD) 174.1 $\pm$ 12.4 U/L in low, 199.1 $\pm$ 18.3 U/L in moderate, and 346.5 $\pm$ 54.2 U/L in high fibrosis stages (P<0.001 for high vs low and moderate); M65: 503.2 $\pm$ 33.1 U/L in low, 635.2 $\pm$ 65.1 U/L in moderate and 988.0 $\pm$ 179.4 U/L in high (P<0.05 for both vs moderate); M65ED: 429.1 $\pm$ 52.4 U/L in low, 549.6 $\pm$ 73.3 U/L in moderate and 1145.5 $\pm$ 224.7 U/L in high (P<0.01 for both vs moderate)	M30: 71% sensitivity and 61% specificity for fibrosis $\geq$ F2 at a cut-off 157.5 U/L; M65: 71% sensitivity and 67% specificity for fibrosis $\geq$ F2 at cut-off 479.5 U/L; M65ED: 74% sensitivity and 68% specificity for fibrosis stages $\geq$ F2 at a cut-off 353.0 U/L; M30: 64% sensitivity and 59% specificity for steatosis >10% at a cut-off 144 U/L; M65: 65% sensitivity and 61% specificity for steatosis >10% at a cut-off 469 U/L; M65ED: 73% sensitivity and 61% specificity for steatosis >10% at a cut-off 310 U/L; M30: 75% sensitivity and 70% specificity for NASH vs NAFLD at cut-off 149.5 U/L; M65: 100% sensitivity and 80% specificity for NASH vs NAFLD at a cut-off 386.0 U/L; M65ED: 100% sensitivity and 80% specificity for NASH vs NAFLD at a cut-off 237.0 U/L
Shen et al (30); 146 patients with biopsy-proven NAFLD (82 NASH) vs 74 healthy controls	CK-18 in serum using M30 ELISA	CK-18: median 103 U/L in controls vs 263 U/L in non-NASH and 418 U/L in NASH (P<0.001)	CD-18: 84.2% sensitivity, 91.9% specificity, 95.4% PPV and 74.7% NPV for NAFLD vs control at cut-off 180 U/L; CD-18: 84.2% sensitivity, 66.2% specificity, 71.16% PPV and 60.2% NPV for NAFLD vs control at a cut-off of 338 U/L
Shen et al (31); 147 patients with biopsy-proven NAFLD vs 74 controls without liver and metabolic diseases	CK-18 in serum using M30 ELISA; CK-18 in serum using M65 ELISA; CK-18 in serum using M65ED ELISA	M30: median 354 U/L (IQR 221–529) in NAFLD vs 103 U/L (IQR 80–138) in controls (P<0.001); M65: median 770 U/L (IQR 539–1010) in NAFLD vs 309 U/L (IQR 249–411) in controls (P<0.001); M65ED: median 443 U/L (IQR 202–801) in NAFLD vs 47 U/L (IQR 30–92) in controls (P<0.001); M30: median 277 U/L (IQR 186–472) in non-NASH vs 397 U/L (IQR 264–657) in NASH (P=0.001); M65: median 637 U/L (IQR 457–886) in non-NASH vs 877 U/L (IQR 671–1469) in NASH (P<0.001); M65ED: median 271 U/L (IQR 187–579) in non-NASH vs 572 U/L (IQR 328–1070) in NASH (P<0.001)	M65: differentiated between grade 1 and grade 2 steatosis (P=0.008); M65ED: differentiated between grade 1 and grade 2 steatosis (P=0.001); M30: did not differentiate between grade 1 and grade 2 steatosis (P=0.190); M30: 84.4% sensitivity, 90.4% specificity, 94.7% PPV and 74.2% NPV for NAFLD vs control at a cut-off of 180 U/L; M65: 76.9% sensitivity, 95.9% specificity, 97.4% PPV and 67.3% NPV for NAFLD vs control at cut-off 523 U/L; M65ED: 93.2% sensitivity, 79.5% specificity, 90.2% PPV and 85.3% NPV for NAFLD vs control at cut-off 105 U/L; M30: 66.7% sensitivity, 60.3% specificity, 59.8% PPV and 67.2% NPV for NASH vs non-NASH at cut-off 338 U/L; M65: 62.3% sensitivity, 70.5% specificity, 65.1% PPV and 67.9% NPV for NASH vs non-NASH at a cut-off 790 U/L; M65ED: 79.7% sensitivity, 57.7% specificity, 62.5% PPV and 76.3% NPV for NASH vs non-NASH at a cut-off 309 U/L

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**TABLE 1 – CONTINUED**  
**Biomarkers of apoptosis**

Author (reference); study	Biomarker, matrix, method	Study population, biomarker levels	Associations and diagnostic performance
Feldstein et al (32); 201 children with biopsy-proven NAFLD (140 NASH)	CK-18 in serum using M30 ELISA	CK-18: mean ( $\pm$ SD) 322.1 $\pm$ 104.8 U/L in NASH vs 164.2 $\pm$ 62 U/L in non-NASH (P<0.001)	CK-18: 85.0% sensitivity, 86.9% specificity, 93.7% PPV, 71.6% NPV for NASH at cut-off of 233 U/L
Kim et al (33); 108 patients undergoing liver biopsy for suspected NASH (1 patient [1.0%] NAFLD type 1; 40 [37.0%] NAFLD type 2; 39 [36.1%] NAFLD type 3 and 28 [25.9%] NAFLD type 4); 67 (62.0%) NASH	CK-18 in serum using M30 ELISA	CK-18: mean ( $\pm$ SD) 279.58 $\pm$ 199.20 U/L in NAFLD subtype 1 and 2 vs 512.20 $\pm$ 636.24 U/L in NAFLD subtype 3 and 4 (P=0.010)	CK-18: 69.0% sensitivity, 64.9% specificity, 75.5% PPV and 57.1% NPV for NASH at cut-off of 235.5 U/L
Cusi et al (34); 424 overweight subjects (300 with NAFLD according to biopsy or magnetic resonance imaging and spectroscopy)	CK-18 in plasma using M30 ELISA	CK-18: 211 U/L (IQR 141–338) in NAFLD vs 148 U/L (IQR 110–195) in non-NAFLD (P<0.0001); CK-18: 179 U/L (IQR 121–231) in simple steatosis vs 122 U/L (IQR 98–155) in non-NAFLD (P<0.0001); CK-18: 232 U/L (IQR 151–387) in NASH vs 170 U/L (IQR 135–234) in simple steatosis (P<0.0001)	CK-18: 63% sensitivity, 83% specificity, 95% PPV and 31% NPV for NAFLD with a cut-off of 165 U/L; CK-18: 58% sensitivity, 68% specificity, 75% PPV and 49% NPV for NASH with a cut-off of 212 U/L; CK-18: 54% sensitivity, 85% specificity, 83% PPV and 56% NPV for fibrosis with a cut-off of 262 U/L
Grigorescu et al (35); 79 patients with biopsy proven-NAFLD (20 non-NASH and 59 NASH)	CK-18 in serum using M65 ELISA	CK-18: mean ( $\pm$ SD) 341.6 $\pm$ 113.2 U/L in non-NASH and 682.4 $\pm$ 451.6 U/L in NASH (P=0.001)	CK-18 M65: 79.6% sensitivity, 66.7% specificity, 86.8% PPV and 53.8% NPV for NASH at cut-off of $\geq$ 340 U/L
Pirvulescu et al (36); 60 overweight, obese or morbidly obese NAFLD patients (47 non-NASH; 13 biopsy-proven NASH)	CK-18 in serum using M30 ELISA; CK-18 in serum using M65 ELISA	M30: mean ( $\pm$ SD) 134.8 $\pm$ 47.3 U/L in non-NASH vs 203.3 $\pm$ 106.0 U/L in NASH (P=0.0037); M65: 282.3 $\pm$ 169.9 U/L in non-NASH vs 597.7 $\pm$ 427.7 U/L in NASH (P=0.0004)	M30: 69.2% sensitivity, 65.2% specificity, 36.0% PPV and 88.2% NPV for NASH vs non-NASH at cut-off of 136 U/L; M65: 69.2% sensitivity, 71.7% specificity, 40.9% PPV and 89.2% NPV for NASH vs non-NASH at cut-off of 389 U/L

CK Cytokeratin; IQR Interquartile range; NAFLD Nonalcoholic fatty liver disease; NASH Nonalcoholic steatohepatitis; NPV Negative predictive value; PPV Positive predictive value; sFas Soluble Fas; vs Versus

identification of a caspase-cleaved fragment of CK-18. In contrast, both M65 (M6 capture and M5 detection antibodies) and M65ED (M5 capture and M6 detection antibodies) assays detect total cell death through the identification of both caspase-cleaved and uncleaved CK-18 (29). Measuring serum biomarkers of cell death is considered to be a noninvasive assessment of fibrosis stages in patients with chronic liver disease because apoptosis is involved in liver fibrosis. While CK-18 M30 was unable to differentiate between low and moderate fibrosis stages, CK-18 M65 and CK-18 M65ED showed better predictive value for fibrosis scores  $\geq$  2. All three markers functioned similarly in predicting precirrhotic fibrosis scores  $\geq$  5. Overall, all three assays could be used effectively to differentiate between NAFLD and NASH; however, only the CK-18 M65 and CK-18 M65ED assays could differentiate between NAFLD and healthy controls (29).

CK-18 M30 was repeatedly found to differentiate between NASH and non-NASH in NAFLD samples. While a clear trend was observed for higher CK-18 levels in patients with more advanced stages of inflammation, steatosis and fibrosis, CK-18 M30 was repeatedly found to be less sensitive, with its usefulness limited by an inability to adequately differentiate between healthy individuals and NAFLD patients. In a large sample of overweight/obese subjects, Cusi et al (34) found significant differences in plasma CK-18 levels using M30 ELISA among all of non-NAFLD, simple steatosis and NASH patients. Differences between controls and non-NASH patients were also found in some smaller samples (26,30,31), while they were absent in other samples (25,29). CK-18 M30 further failed to differentiate between fibrosis stages 1 and 2. In the same study, M65 and M65ED could be used to predict fibrosis stages 1 or 2 (31).

Two models combining CK-18 and hyaluronic acid, and CK-18 and soluble Fas, respectively, showed better predictive value for NASH versus non-NASH in patients with NAFLD (27,28). Soluble Fas ligand was elevated in NAFLD patients with fibrosis compared

with NAFLD without fibrosis (37). Elsewhere, double immunohistochemistry staining for CK-8/18 and ubiquitin improved detection of hepatocyte damage in NAFLD. This assay was used to detect ballooned hepatocytes, because ballooned hepatocytes lack CK-8/18 immunostaining on biopsy. Staining was correlated with fibrosis stage, especially advanced fibrosis, and steatohepatitis in 40 adult NAFLD core liver biopsies (38).

The number of hepatic progenitor cells and ductular reaction, assessed by CK-7 immunostaining, was used as an indicator of different fibrosis patterns in 38 pediatric NASH patients (39). Although the incidence of CK-7-positive centrilobular hepatocytes using immunostaining was 64.3% in 14 needle biopsy liver specimens belonging to NASH patients, CK-7 immunostaining was not associated with the stage of fibrosis or the grade of steatosis. CK-7 immunostaining was more common in patients with elevated serum AST and GGT (40).

Significantly higher caspase 3 and 8 activity was observed in patients with NASH than in simple steatosis in a small sample of 50 NAFLD patients. Mean caspase 3 and 8 activity scores were comparable between patients with normal and patients with elevated ALT levels (41).

### ADIPOKINES

The main adipokines and cytokines involved in the pathogenesis of NAFLD include adiponectin, leptin, resistin, visfatin, TNF-alpha and interleukin (IL)-6 (42). Adiponectin, a 30 kDa protein, is exclusively synthesized by adipose tissue and has roles in glucose and lipid metabolism (43,44). Its secretion is stimulated by insulin and is induced during fat cell differentiation. Adiponectin modifies insulin receptor function and influences hepatocellular free acid metabolism. Circulating levels of adiponectin are negatively associated with insulin resistance, type 2 diabetes and dyslipidemia (45-57). Table 2 details findings of recent studies with respect to adiponectin (34-36,58-65) and leptin (36,63-66) levels in NAFLD patients.

**TABLE 2**  
**Studies investigating adiponectin, leptin, resistin and retinol binding protein 4 in NAFLD patients**

Author (reference); study	Biomarker, matrix, method	Study population levels of biomarker	Associations and diagnostic performance
Cusi et al (34); 424 overweight subjects (300 with NAFLD according to biopsy or magnetic resonance imaging and spectroscopy)	Adiponectin in plasma by ELISA	Adiponectin: mean ( $\pm$ SD) 8.1 $\pm$ 0.3 $\mu$ g/mL in NAFLD vs 14.3 $\pm$ 1.0 $\mu$ g/mL in non-NAFLD (P<0.001)	
Grigorescu et al (35); 79 patients with biopsy proven-NAFLD (20 non-NASH and 59 NASH)	Adiponectin in serum using ELISA	Adiponectin: mean ( $\pm$ SD) 8.714 $\pm$ 5.740 $\mu$ g/mL in non-NASH and 4.333 $\pm$ 2.031 $\mu$ g/mL in NASH (P<0.001)	Adiponectin: 86.2% sensitivity, 61.9% specificity, 86.2% PPV and 61.3% NPV for NASH at a cut-off of $\leq$ 6.0 $\mu$ g/mL
Pirvulescu et al (36); 60 overweight or morbidly obese patients with significant weight-related comorbidities (13 NASH and 47 non-NASH)	Adiponectin in serum using ELISA; leptin in serum using ELISA	Adiponectin: mean ( $\pm$ SD) 18.5 $\pm$ 6.0 $\mu$ g/mL in non-NASH vs 9.2 $\pm$ 3.7 $\mu$ g/mL in NASH (P=0.00001); leptin: mean ( $\pm$ SD) 34.1 $\pm$ 31.1 ng/mL in non-NASH vs 59.8 $\pm$ 32.9 ng/mL in NASH (P=0.006)	Adiponectin: 92.3% sensitivity, 86.7% specificity, 66.7% PPV and 97.5% NPV to discriminate non-NASH from NASH at a cut-off of 13.5 $\mu$ g/mL; leptin: independent predictor of NASH (OR 3.09 [95% CI 0.86–11.12], P=0.00001); leptin: 61.5% sensitivity, 65.9% specificity, 34.8% PPV and 85.3% NPV to discriminate non-NASH from NASH at a cut-off of 40 ng/mL
Balmer et al (58); 64 NAFLD, 123 other chronic liver disease, 45 cirrhosis and 20 healthy controls	Serum adiponectin using ELISA	Adiponectin: mean ( $\pm$ SD) 18.6 $\mu$ g/mL in cirrhosis, 4.8 $\mu$ g/mL in NAFLD, 10.4 $\mu$ g/mL in other liver diseases and 9.1 $\mu$ g/mL in controls	Adiponectin: decreased in NAFLD compared with other liver disease (4.8 $\pm$ 3.5 $\mu$ g/mL vs 10.4 $\pm$ 6.3 $\mu$ g/mL, P<0.0001); Adiponectin: correlated with body weight and serum triglycerides levels (P<0.001)
Gastaldelli et al (59); 47 NASH patients vs 20 controls without typical risk factors for NASH	Plasma adiponectin using radioimmunoassay	Adiponectin in controls: mean ( $\pm$ SD) 14.1 $\pm$ 1.7 $\mu$ g/mL in non-obese vs 12.5 $\pm$ 2.1 $\mu$ g/mL in obese; adiponectin in NASH: 6.8 $\pm$ 1.1 $\mu$ g/mL in non-obese vs 6.4 $\pm$ 0.5 $\mu$ g/mL in obese (P<0.05 vs controls)	
Leite et al (60); 84 type 2 diabetes with biopsy-proven NAFLD	Adiponectin in plasma using immunoassay	Adiponectin: median 12.77 $\mu$ g/mL (IQR 0.13–1773.05) in non-NASH and 8.59 $\mu$ g/mL (IQR 1.66–33.14) in NASH (P=0.015)	Adiponectin: OR 0.92 (95% CI 0.84–0.99), P=0.045 for NASH; adiponectin: OR 0.87 (95% CI 0.79–0.96), P=0.006 for fibrosis
Ozcelik et al (61); 129 patients: HOMA-IR $\geq$ 2.5 and grade 0 steatosis or HOMA-IR <2.5 and grade 1–2 steatosis (group I); 145 patients: HOMA-IR $\geq$ 2.5 and grade 1–2 steatosis or HOMA-IR <4 and grade 3 steatosis (group II); 41 patients: HOMA-IR $\geq$ 4 and grade 3 steatosis (group III)	Serum adiponectin using ELISA	Adiponectin: mean ( $\pm$ SD) 13.6 $\pm$ 3.3 $\mu$ g/mL in group I, 12.4 $\pm$ 3.7 $\mu$ g/mL in group II (P<0.05 vs group I); 11.6 $\pm$ 3.5 $\mu$ g/mL in group III (P<0.01 vs group I)	Significant inverse relationship between adiponectin and insulin resistance (P<0.0001), insulin level (P<0.0001), glucose level (P<0.0001), hemoglobin A1c blood glucose level (P<0.0001), triglycerides level (P<0.0001) and the degree of hepatosteatosis (P<0.0001)
Xu et al (62); 85 ultrasonography-proven NAFLD patients (45 euglycemic and 40 hyperglycemic) vs 35 healthy controls	Serum adiponectin using ELISA	Adiponectin: median 2.25 $\mu$ g/mL (IQR 1.42–3.30) in euglycemic NAFLD patients vs 7.70 $\mu$ g/mL (IQR 5.42–9.98) in controls (P<0.001); adiponectin: 1.55 $\mu$ g/mL (IQR 1.08–2.43) in hyperglycemic NAFLD patients vs 2.25 $\mu$ g/mL (IQR 1.42–3.30) in euglycemic NAFLD patients (P<0.05)	Inverse relationship between adiponectin levels and hyperglycemia in the NAFLD sample (OR 0.654 [95% CI 0.431–0.993], P=0.046)
Zelber-Sagi et al (63); 338 individuals in the general population with liver diseases: 106 (31.4%) steatosis; 3 (0.9%) NASH; 229 (67.7) non-NASH vs controls. Fibrosis diagnosed using FibroTest* in 87 (25.7%)	Adiponectin in serum using ELISA; leptin in serum using ELISA	Adiponectin in men: mean ( $\pm$ SD) 10.2 $\pm$ 6.2 $\mu$ g/mL in control vs 7.1 $\pm$ 4.7 $\mu$ g/mL in NASH (P<0.001); adiponectin in women: 17.4 $\pm$ 12.1 $\mu$ g/mL in control vs 10.1 $\pm$ 5.4 $\mu$ g/mL in NASH (P<0.001). Leptin in men: 7.8 $\pm$ 10.4 ng/mL in control vs 13.4 $\pm$ 11.3 ng/mL in NASH (P=0.001); leptin in women: 4.8 $\pm$ 17.1 ng/mL in control vs 43.8 $\pm$ 18.8 ng/mL in NASH (P<0.001)	Adiponectin: OR 0.92 (95% CI 0.86–0.98), P=0.01 for NASH or borderline NASH; leptin: OR 1.03 (95% CI 1.01–1.06), P=0.003 for NASH or borderline NASH; suboptimal adiponectin and elevated leptin: OR 20.7 (95% CI 7.5–57.5) for NASH or borderline NASH
Koehler et al (64); Obese patients: 72 NASH (60 NASH with fibrosis stage $\leq$ 1, 12 NASH with fibrosis stage $\geq$ 2) and 88 non-NASH (16 normal liver histology, 72 patients with simple steatosis)	Adiponectin in serum using ELISA; leptin in serum using ELISA; resistin in serum using ELISA	Adiponectin: median 12.909 $\mu$ g/mL (IQR 8.050–19.292) in control, 8.563 $\mu$ g/mL (IQR 6.261–12.063) in simple steatosis, 6.833 $\mu$ g/mL (IQR 5.035–11.513) in NASH with FS $\leq$ 1 and 3.930 $\mu$ g/mL (IQR 3.235–6.453) in NASH with FS $\geq$ 2 (P<0.0001 for NASH vs non-NASH); leptin: median 40.6 ng/mL (IQR 32.0–60.5) in controls, 44.6 ng/mL (IQR 28.4–60.4) in simple steatosis, 41.0 ng/mL (IQR 32.3–56.0) in NASH with FS $\leq$ 1 and 48.3 ng/mL (IQR 38.8–70.1) in NASH with FS $\geq$ 2; resistin: median 15.1 ng/mL (IQR 13.7–23.4) in controls, 15.3 ng/mL (IQR 12.7–19.0) in steatosis, 14.9 ng/mL (IQR 12.6–17.8) in NASH with FS $\leq$ 1 and 17.5 ng/mL (IQR 14.2–19.7) in NASH with FS $\geq$ 2	Adiponectin: 71.4% specificity, 39.4% sensitivity, 64.2% PPV and 68.2% NPV for differentiating non-NASH from NASH at cut-off of 7.149 $\mu$ g/mL; adiponectin: 63% sensitivity and 94% specificity for differentiating mild NASH from advanced NASH at cut-off of 4.080 $\mu$ g/mL; leptin and resistin not significant predictors of NASH vs non-NASH

Continued on next page

**TABLE 2 – CONTINUED**  
**Studies investigating adiponectin, leptin, resistin and retinol binding protein 4 in NAFLD patients**

Author (reference); study	Biomarker, matrix, method	Study population levels of biomarker	Associations and diagnostic performance
Polyzos et al (65); 24 lean controls, 28 obese controls, 15 NAFLD patients, 16 NASH patients	Adiponectin in serum using immunoassay; leptin in serum using ELISA; RBP4 in serum using ELISA	Adiponectin: mean ( $\pm$ SD) 15.7 $\pm$ 1.1 $\mu$ g/mL in lean, 11.1 $\pm$ 0.8 $\mu$ g/mL in obese ( $P$ <0.05 vs lean), 8.1 $\pm$ 1.3 $\mu$ g/mL in NAFLD and 4.6 $\pm$ 0.7 $\mu$ g/mL in NASH ( $P$ <0.05 vs lean, $P$ <0.05 vs obese); leptin: 10.2 $\pm$ 1.5 ng/mL in lean, 17.0 $\pm$ 2.5 ng/mL in obese, 20.1 $\pm$ 4.0 ng/mL in NAFLD and 32.2 $\pm$ 5.7 ng/mL in NASH ( $P$ <0.05 vs lean, $P$ <0.05 vs obese); RBP4: 0.59 $\pm$ 0.06 $\mu$ g/mL in lean, 0.85 $\pm$ 0.10 $\mu$ g/mL in obese, 0.61 $\pm$ 0.10 $\mu$ g/mL in NAFLD and 0.64 $\pm$ 0.13 0.14 $\mu$ g/mL in NASH	Serum leptin and adiponectin correlated with insulin resistance and cardiometabolic factors
Medici et al (66); 104 diabetic morbidly obese NAFLD patients	Plasma leptin using radioimmunoassay	Leptin: mean ( $\pm$ SD) 43.7 $\pm$ 20.1 ng/mL in morbidly obese patients; soluble leptin receptor: 38.4 $\pm$ 28.4 U/mL in morbidly obese patients; free leptin index: 1.5 $\pm$ 1.1 ng/U in morbidly obese patients	Plasma leptin associated with body mass index ( $P$ <0.0001). No association between steatosis and circulating leptin concentrations. No association between fibrosis and circulating leptin concentrations

\*LabCorp, USA. ELISA Enzyme-linked immunosorbent assay; FS Fibrosis score; HOMA-IR Homeostasis model assessment of insulin resistance; IQR Interquartile range; NAFLD Nonalcoholic fatty liver disease; NASH Nonalcoholic steatohepatitis; NPV Negative predictive value; PPV Positive predictive value; RBP4 Retinol binding protein 4; vs Versus

Mean ( $\pm$  SD) adiponectin levels were low (6.88 $\pm$ 4.50  $\mu$ g/mL) in a small sample of 12 NAFLD patients (67). Serum adiponectin levels were lower in patients with NASH than in those without NASH in small samples of patients with biopsy-proven NAFLD (33,35,60,64,68). Based on findings from a recent meta-analysis (69), adiponectin is a biomarker of NAFLD progression to NASH because differences in serum adiponectin levels between controls and NAFLD patients are less pronounced. Adiponectin is a negative predictor of NASH in NAFLD patients at a cut-off of 29.16  $\mu$ g/L (70). Furthermore, adiponectin levels were decreased in NAFLD patients compared with patients with other chronic liver conditions (58). An inverse relationship with fibrosis was also found (60). Adiponectin was not a predictor of either steatosis or fibrosis in a small pediatric NAFLD sample (25).

In a sample of 35 healthy controls and 85 ultrasonography-proven NAFLD patients (45 euglycemic and 40 hyperglycemic), serum adiponectin levels were significantly lower in euglycemic NAFLD patients than in controls, and in hyperglycemic compared with euglycemic NAFLD patients. A significant inverse relationship was further found between adiponectin levels and hyperglycemia in the NAFLD sample as a whole (62). Hypoadiponectinemia in NAFLD is part of the metabolic disturbances associated with MS, with a significant inverse relationship found between serum adiponectin levels and hepatic fat content (71). A significant inverse relationship was found between serum adiponectin levels and all of insulin resistance, insulin level, glucose level, hemoglobin A1c blood glucose level and triglycerides level in a large sample of obese male patients. Furthermore, adiponectin levels were higher in individuals with lower hepatosteatosis (61,65).

NASH was associated with an increase in the messenger RNA expression of the adiponectin receptor ApoR2, which was further associated with hepatocellular apoptosis (70). NAFLD progression in morbid obesity is associated with an increase in hepatic adiponectin receptors expression (72).

An inverse correlation was found between adiponectin and bile acids, while a trend was shown between bile acid levels and NASH. This suggests that adiponectin may be protective through its effect on bile acid metabolism (70).

Based on large variations in mean adiponectin levels found in different studies, this adipokine appears unlikely to be useful as a sole diagnostic biomarker in NAFLD/NASH. However, its regular monitoring can aid in assessing NAFLD progressing or regression over time in the same patient. For example, Wong et al (73) found that adiponectin levels increased after a three-year follow-up compared with baseline in NAFLD patients who received dietary counselling and were encouraged to increase physical activity. However, changes in

adiponectin levels over this period were not correlated with increased, static or decreased NAFLD activity score (73).

Another prominent adipocytokine associated with NAFLD is leptin. Leptin is a 16 kDa proteohormone that is believed to be involved in the regulation of food intake, energy balance and body weight by increasing energy expenditure (74,75). Leptin is synthesized by differentiated adipocytes but has also been documented in other tissues including skeletal muscle, liver and the placenta (76-79). Leptin concentrations in the blood reflect total body fat (80).

Leptin levels were higher in an obese pediatric population with concomitant NAFLD than in obese control patients without steatosis (68), and in patients with more advanced fibrosis in a small pediatric NAFLD sample (25). Serum leptin was further associated with insulin resistance (65,81). A model combining serum suboptimal adiponectin and elevated leptin was used to predicted NASH or borderline NASH (63).

Increases in leptin levels and decreases in adiponectin levels occurred similarly in patients with simple steatosis or NASH during obesity reversal after bariatric surgery, suggesting that these changes are due to morbid obesity and occur independent of liver disease (82). In a separate study, leptin and adiponectin levels remained independent predictors for NASH in obese patients (36). Similarly, progressive leptin increases and progressive adiponectin decreases occurred with increasing steatosis severity. Leptin increases were further correlated with fibrosis (83). Both leptin and adiponectin were associated with NASH in another study. However, the association between these adipokines and fibrosis assessed by FibroTest (LabCorp, USA) was no longer present (63).

Ghrelin is a 28 amino acid peptide, produced mainly by the stomach, which plays a major role in energy balance (84,85). However, small amounts of ghrelin are synthesized in the hypothalamus, pituitary, bowel, placenta and kidney (85,86). Ghrelin stimulates lactotroph and corticotroph secretion, and influences gastroenteropancreatic function and insulin secretion, as well as glucose and lipid metabolism. In addition, ghrelin has cardiovascular and antiproliferative effects (87,88).

Ghrelin has adipogenic properties (85). Ghrelin levels correlate negatively with insulin resistance and hyperinsulinemia, and contribute to the feedback mechanism by which body weight is regulated (89). Low levels of ghrelin are found in diabetic children, which may represent a defense mechanism against hyperglycemia (90,91).

Serum levels of retinol binding protein 4 (RBP4), another adipokine associated with insulin resistance, were also higher in NAFLD patients compared with controls (68). Adiponectin and resistin levels were negatively correlated with leptin and RBP4 levels (68). Based on

**TABLE 3**  
**Noninvasive markers to differentiate disease progression correlated with the gold standard**

Correlation	Nonspecific, nonsensitive	Specific, sensitive
Slight correlation with severity of inflammation NAFLD/NASH; no correlation with fibrosis	Age, body mass index, insulin resistance, AST/ALT, platelet count, albumin, haptoglobin, $\alpha$ 2-macroglobulin, bilirubin, $\gamma$ -glutamyl transpeptidase	
Correlation with severity of metabolic syndrome NAFLD/NASH; no correlation with fibrosis or inflammation	Dyslipidemia: triglycerides, cholesterol, HDL, apolipoprotein A1, apolipoprotein B	
Oxidative stress correlated with inflammation steatosis and ballooning		Linoleic acid oxidation product: 13-hydroxy-octadecadienoic acid
Adipokine (adipocyte hormone), adiponectin, lower levels in NASH than in NAFLD with simple steatosis		Adiponectin
Adipokine, leptin, higher levels in obese NAFLD patients than in obese patients without steatosis		Leptin
Progression of inflammation		Ghrelin, ubiquitin sensitive markers for NASH
Sensitive inflammation markers		TNF- $\alpha$ , interleukins (IL-6; IL-8), RANTES and Fas ligand
Markers of liver fibrosis may help predict the evolutionary course of NAFLD		Hyaluronic acid, procollagen III N-terminal peptide, TGF- $\beta$ and TIMP1
Cytokeratin-18 fragment: M30 – higher in NASH than in NAFLD with simple steatosis, correlation with inflammation, steatosis and fibrosis, no difference between healthy individuals and NAFLD patients; M65 – better predicating fibrosis F $\geq$ 2		Markers of cell death CK-18 by apoptosis (fragment M30) and necrosis (M65)
Mitochondrial dysfunction cytochromes: provide powerful predictions of risk in NASH		CK-7/CK-18

ALT Alanine aminotransferase; AST Aspartate aminotransferase; CK Cytokeratin; F1-F4 Fibrosis scores 1 to 4; HDL High-density lipoprotein; RANTES Regulated on activation, normal T cell expressed and secreted; TIMP Tissue inhibitor of metallo-protease; TGF- $\beta$  Transforming growth factor-beta; TNF- $\alpha$  Tumour necrosis factor-alpha

these findings, all of adiponectin, leptin, resistin, ghrelin and retinol RBP4 may be appropriate biomarkers of NAFLD or MS, but are unlikely to serve as diagnostic markers on their own.

### CYTOKINES AND INFLAMMATORY MARKERS

Inflammatory cytokines and chemokines used to diagnose NASH include TNF-alpha and IL-6, the chemokine CC-chemokine ligand-2 (chemo-attractant protein-1), as well as the inflammation marker high-sensitivity C-reactive protein (hs-CRP) (24). Suppression of cytokine signalling-3 downregulates hepatocellular insulin receptors and promotes acquired hepatic insulin resistance (12).

Several recent studies measured inflammatory markers in NAFLD patients (36,60,92-95). Two of these studies compared NAFLD patients with non-NAFLD controls (92,94), while the remaining three compared NASH patients with non-NASH patients in NAFLD cohorts (36,60,95). TNF-alpha was higher in NAFLD patients compared with non-NAFLD controls (92,94). It was also higher in NASH compared with non-NASH in one NAFLD sample (95), while it was comparable between NASH and non-NASH in another (36). IL-6 was higher in NAFLD patients compared with non-NAFLD controls (94), with no differences between NASH and non-NASH (36,95). Another marker of inflammation, hs-CRP, was higher in NAFLD patients compared with non-NAFLD controls (92,94). It was also higher in NASH compared with non-NASH in one NAFLD sample (36), while it was comparable between NASH and non-NASH in other NAFLD samples (93, 95). TNF-alpha, IL-6, IL-8, IL-10, hs-CRP and TNF-beta were similar between NASH and non-NASH, and hence not associated with NASH in a sample of NAFLD patients (60).

### CLINICAL PARAMETERS

Several studies assessed biochemical parameters in NAFLD patients and healthy controls (30,92,94,96-108). In these studies, body mass index or waist circumference, AST, ALT, GGT, triglycerides, glucose, insulin and the homeostasis model assessment of insulin resistance were either significantly higher or showed a trend toward higher levels in NAFLD patients compared with healthy controls. Total cholesterol and

low-density lipoprotein levels were similarly higher and high-density lipoprotein levels were lower, with trends observed in smaller samples (30,92,94,96-108). Diabetes mellitus, MS and hypertension were significantly more predominant in NAFLD patients than in controls in these samples (30,92,98-101,103,107). Differences between NAFLD and controls without liver injury were further assessed in obese patients or patients with coronary artery disease (109-112). Similar patterns were observed in obese patients and normal weight patients with respect to metabolic parameters in the presence or absence of NAFLD (109-111). In contrast, metabolic parameters were comparable between NAFLD and normal patients with coronary artery disease (112).

Metabolic profiles were further compared between individuals with simple steatosis and patients with NASH in NAFLD samples. These parameters were often comparable between simple steatosis and NASH, although similar trends as those observed between controls and NAFLD were maintained between simple steatosis and NASH (30,33,35,36,95,113-119). Similarly, the incidence of MS, hypertension and diabetes mellitus was comparable between individuals with simple steatosis and patients with NASH in NAFLD samples (35).

Table 3 illustrates the sensitivity of different biomarkers to differentiate the possible progression of liver damage in NAFLD/NASH.

Markers of MS cannot be used alone to predict NAFLD/NASH, but their periodic monitoring offers an avenue to predict those patients that carry an increased risk of developing NAFLD/NASH, or those at increased risk for disease progression or extrahepatic manifestations.

### METABOLOMICS AND PROTEOMICS

Metabolomics and proteomics are some of the techniques used to identify new markers of NAFLD/NASH. The compounds whose serum levels were observed to differ between patients with NASH and non-NASH controls could be associated with biochemical perturbations associated with liver dysfunction (eg, reduced creatinine) and inflammation (eg, eicosanoid signalling) in an exploratory metabolomics approach using ultra performance liquid chromatography-mass spectrometry (120).

Metabolomics analysis revealed that NAFLD was associated with higher plasma concentrations of homocysteine and total cysteine and

lower plasma concentrations of total glutathione than controls in nondiabetic subjects with hepatic steatosis or NASH. Markedly higher levels of glycocholate, taurocholate and glyco-cheno-deoxycholate were further observed in subjects with NAFLD. Plasma concentrations of long-chain fatty acids were lower, while those of free carnitine, butyrylcarnitine and methylbutyrylcarnitine were higher in NASH. Several glutamyl dipeptides were also higher and cysteine-glutathione levels were lower in NASH and steatosis (96). Elsewhere, serum metabolomics revealed levels of gamma-Glu-Val, gamma-Glu-Thr, gamma-Glu-Leu, gamma-Glu-His, gamma-Glu-Phe and gamma-Glu-Arg were higher in simple steatosis than in NASH (121). Gamma-glutamyl dipeptides, biosynthesized through a reaction with gamma-glutamylcysteine synthetase, indicated production of reduced glutathione (121). Using a metabolomics approach, Li et al (122) further identified serum glucose, glutamate, lactate and taurine levels as biomarkers of NAFLD and constructed a model to predict the different stages of NAFLD progression based on the different levels of these biomarkers in each individual.

A proteomics analysis revealed that the metabolic profile was different depending on an individual's level of obesity. This analysis was useful in distinguishing steatosis from NASH in a sample of patients with biopsy-proven NAFLD (123). Proteomics analysis was used to classify the serum protein fingerprint of NAFLD in 35 NAFLD patients and 35 healthy controls. Hemoglobin subunit  $\alpha$  was upregulated in NAFLD compared with controls. High baseline hemoglobin levels predicted an increased risk of developing NAFLD during a three-year follow-up period in a large sample of previously healthy individuals (124).

Using two distinct shotgun proteomic techniques, Miller et al (125) found that several proteins are up/downregulated in NAFLD compared with healthy controls. Of these, afamin, apolipoprotein E, CD5 molecule-like, complement C3, insulin-like growth factor-binding protein 3, vitamin D-binding protein and lymphocyte cytosolic protein 1 were upregulated in serum sample of patients with NAFLD, compared with controls (125). Furthermore, apolipoprotein E, catalase, CD5 molecule-like, lymphocyte cytosolic protein 1 and vitamin D-binding protein were upregulated in serum sample of patients with NASH compared with those with simple steatosis (125).

A recent review by Naik et al (126) discusses the association between NAFLD and genetic variants in different ethnic groups in patatin-like phospholipase domain containing 3, manganese superoxide dismutase (SOD<sub>2</sub>), TNF- $\alpha$ , glutathione S-transferases, peroxisome-proliferator-activated receptor- $\alpha$  signalling, microsomal triglyceride transfer protein, phosphatidyl-ethanolamine methyltransferase, apolipoprotein C-3, apolipoprotein E, adiponectin signalling, insulin receptor signalling, growth hormone and insulin-like growth factor axis, IL-6, as well as drug-metabolizing enzymes.

### MARKERS OF HEPATIC FIBROSIS

A strong correlation was found between NASH, diabetes mellitus and fibrosis in a large sample of overweight patients (127). Several noninvasive markers of fibrosis have been used in NAFLD patients, including NAFLD fibrosis score, AST/platelet ratio index, FIB-4 score and BARD score. These markers can help predict those patients who would be at highest risk of developing liver-related complications or death (128-130). Advanced fibrosis, inferred from higher scores in these assays, was associated with liver-related adverse events and shorter cumulative survival. Liver-related outcomes were not associated with the degree of steatosis or the presence of NASH, indicating the necessity to adequately assess liver fibrosis, using noninvasive markers, to predict outcomes in NAFLD patients. All four assays successfully differentiated between patients with low risk and patients with higher risk, for both liver-related adverse events and cumulative survival (129). These markers were also used elsewhere to characterize fibrosis (131). These noninvasive fibrosis scoring systems had good negative predictive value but poor positive predictive value, suggesting that they can be used to exclude fibrosis in NAFLD patients and, hence, prevent biopsy in patients without fibrosis (132).

De Lédighen et al (133) describe a novel physical parameter based on the properties of ultrasonic signals acquired by the FibroScan (Echosens, France). This test, implemented together with FibroScan, can be used with a high degree of sensitivity to simultaneously assess fibrosis and steatosis.

FibroTest is a commercial algorithm based on different biochemical markers used to assess and monitor liver fibrosis progression (134,135). FibroTest further characterized fibrosis in samples of obese patients, identifying those patients with advanced fibrosis and, thus, in need of biopsy (136).

Neuman et al (12) showed that transforming growth factor- $\beta$  correlation with fibrosis stages F1 to F3 is strong. However, in patients with cirrhosis, this marker drops significantly because the stellate cells have been reduced (12).

Liver stiffness measurement estimated fibrosis in NAFLD patients. However, no differences were found between stages 1 and 2 of fibrosis (137). Both the pediatric NAFLD fibrosis index and transient elastography were higher in individuals with significant fibrosis in 67 children with biopsy-proven NAFLD (10 with fibrosis stage  $\geq 2$  and 57 with fibrosis stage  $< 2$ ) (138). Combining the two models yielded a cut-off of 8.2 for the pediatric NAFLD fibrosis index, below which clinically significant fibrosis can be ruled out. A pediatric NAFLD fibrosis index score of  $\geq 8.2$  led to transient elastography, and a transient elastography score of  $< 8.6$  kPa further revealed early liver fibrosis while a transient elastography score of  $\geq 8.6$  kPa predicted significant fibrosis with 100% accuracy and, hence, a need for liver biopsy (138).

### OXIDATIVE STRESS

A strong correlation was found between oxidative stress markers and insulin resistance in obese adolescents with NAFLD (139). Oxidative stress, as indicated by higher plasma reactive carbonyl species levels, may be a direct risk factor for developing NAFLD (140).

Oxidative stress was assessed based on the balance of SOD<sub>2</sub>, an enzyme with antioxidant activity, and cytochrome p450 2E1 (CYP2E1), an enzyme with pro-oxidant activity, based on data from 100 NASH patients, 31 simple steatosis patients and 90 healthy controls. SOD<sub>2</sub> 47T>C and CYP2E1 1053C>T variants were genotyped using polymerase chain reaction. While the distribution of genetic variants was not different among groups for either enzyme, the presence of the higher activity SOD<sub>2</sub> C allele was higher in NASH patients compared with the other two groups (141).

CYP2E1 plays an important role in fatty acid metabolism, and it leads to the formation of toxic lipid peroxides. A recent study showed that lipid peroxidation was significantly greater in biopsies obtained from pediatric NAFLD patients than in patients with normal liver histology ( $P < 0.001$ ), without any significant differences in hepatic CYP2E1 expression. Furthermore, lipid peroxidation and CYP2E1 protein content were comparable between patients with simple steatosis and NASH patients (142). Thus, CYP2E1 may be responsible for reactive oxygen species overproduction, which plays an important role in the progression of NAFLD to NASH. While CYP2E1 expression profiles may not differ between simple steatosis and NASH, high CYP2E1 activity may be associated with progression to NASH. Furthermore, increased CYP2E1 expression may be an adaptive mechanism to prevent lipid overload. Recent evidence further shows a correlation between CYP2E1 activity, reactive oxygen species overproduction, mitochondrial assembly of reactive oxygen species and insulin resistance. These effects are detailed in recent reviews (143-145).

In addition, alcohol consumption was found to be a risk factor for NASH, because increased expression and activity of the pathways for alcohol catabolism, including CYP2E1, alcohol dehydrogenase and aldehyde dehydrogenase was found in liver tissues of NASH patients compared with normal controls, suggesting increased scavenging of alcohol from circulation in individuals with NASH (146).

Dietary long-chain fatty acids are an additional source of oxidative stress in NAFLD/NASH. Long-chain fatty acids represent normal

intermediates in fat metabolism. Depending on the hepatocellular capacity to metabolize free fatty acids, the concentration of long-chain fatty acids and that of their activated form, acyl-coenzyme A, may be lipotoxic. Free fatty acids damage hepatic mitochondria by down-regulating their beta oxidation and producing oxidative stress. Furthermore, accumulation of ceramides and diacylglycerol may decrease triglyceride to fatty acid ratio leading to lipotoxicity (147).

### CONCLUSION

Unfortunately, to date, many critical gaps exist in the reference interval database of most of the biomarkers that have been identified for evaluation of hepatic steatosis. In addition, there are critical differences in our current knowledge of the normal levels of these biomarkers in healthy individuals depending on sex, ethnicity, age, nutritional status and comorbidities. The identification of the patients who are at risk for developing steatohepatitis that will advance to cirrhosis and complications of end-stage liver disease remains a challenge. Noninvasive biomarkers are being developed to replace liver biopsy. Hopefully, these biomarkers will provide accurate and reproducible predictive outcome data. A greater understanding of the evolution of the disease must also evaluate the role of lipid metabolism on all liver cells such as hepatocytes, stellate cells and Kupffer cells. The investigation of biomarkers may lead to therapeutic options that could be used to prevent inflammation and fibrosis in the individuals with fatty liver disease. These biomarkers would be a powerful evaluative tool for monitoring of patients with steatohepatitis. The key to a successful

prevention program will depend on the early identification, treatment and monitoring of high-risk individuals by measuring a number of disease-specific biomarkers including the ones presented in the present review. Biomarkers are essential to screening and treatment strategies for patients with fatty liver disease, and diagnosing patients with life-threatening NAFLD and NASH more quickly. This would enable classification and staging of disease using a simple blood test for biomarkers, thus avoiding a liver biopsy. Managing the underlying comorbidities generating NASH syndrome is achievable and should improve the natural history of this challenging disease. In addition, biomarker strategies can platform the biomedical research.

Moreover, we are convinced that the researchers studying MS, in which category NAFLD/NASH belongs, will have to evaluate not only the liver-related mechanisms, biomarkers and injuries, but also other tissues and organs cross-influencing the liver such as the gut, adipose tissue and the central nervous system.

A team in which the biotechnology industry will work with the researcher, hepatologist, cardiologist, endocrinologist and nutrition specialist may be beneficial for the patient.

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