# Research Progress of Noninvasive Diagnostic Methods for Nonalcoholic Steatohepatitis

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**Abstract** Distinguishing between nonalcoholic steatohepatitis (NASH) and advanced liver fibrosis is the key for clinical diagnosis of non-alcoholic fatty liver disease (NAFLD). Liver biopsy, which is widely used for diagnosis of liver diseases at present, has many drawbacks, such as being invasive, expensive and unstable. This article compares and summarizes the commonly used non-invasive diagnostic methods, including their diagnostic parameters, advantages and disadvantages, in order to provide a useful reference for the diagnosis of NASH.

**Key words** Nonalcoholic steatohepatitis; Liver fibrosis; Non-invasive diagnostic methods; Bio-markers

Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological syndrome characterized by excessive fat deposition in liver cells, caused by factors other than alcohol and other well-defined liver-damaging factors. It is an acquired, metabolic stress related liver injury, closely related to insulin resistance and genetic susceptibility. Approximately 25% of adults worldwide have NAFLD[1], making it the most common chronic liver disease worldwide. As an active form of NAFLD, non-alcoholic steatohepatitis (NASH), is characterized by histological lobular inflammation, hepatocyte ballooning, which is associated with a more rapid progression of fibrosis.

The global prevalence of NASH is estimated to range from 1.5% to 6.5% in adults. As liver fibrosis progresses, collagen deposition and subsequent vascular remodeling manifest as cirrhosis. When NASH progresses to cirrhosis, clinicians are unable to make effective diagnostic and therapeutic interventions before serious liver complications develop, posing a serious threat to patients' lives and health[2-3].

Currently, liver biopsy remains the gold standard for making a definitive diagnosis of NASH. However, the clinical application of this diagnostic method is somewhat limited by its drawbacks such as being invasive, expensive, potential errors caused by sampling and *etc.* Therefore, there is an urgent need for a non-invasive liver biopsy in clinical to facilitate the diagnosis and treatment of NASH. Yet there are few non-invasive diagnostic methods specifically designed to identify high-risk NASH, it is crucial to develop an accurate and effective method to distinguish NASH from liver fibrosis[4].

In this paper, we compare the commonly used non-invasive diagnostic methods of NASH in clinical practice to summarize their advantages and disadvantages, including hepatic fibrosis markers of NASH such as cytokeratin 18 and PRO-C3, diagnostic imaging techniques, and four commonly used scoring systems.

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## Diagnosis Based on Hepatocyte Markers

### Cytokeratin CK18

Cytokeratin 18 (CK18) is the major intermediate filament protein in the hepatic cytoskeleton. It is cleaved and released into the extracellular space at the onset of cell death[5]. Necrosis and apoptosis are two forms of hepatocyte death. Hepatic enzymes are released from hepatocytes undergoing necrosis, and thus elevated glutathione aminotransferase (ALT)/glutathione aminotransferase (AST) can be detected in the blood. During hepatocyte apoptosis, which is typically associated with cell shrinkage, damage associated molecular patterns (DAMPs) are inactivated by autophagy, but cytoskeleton proteins including CK18, M30 and M65 are released. Among them, M30 is a highly specific marker of liver apoptosis, and M65 reflects the extent of cell death (apoptosis and necrosis).

Both M30 and M65 are valuable in the diagnosis of NASH (pooled AUROC: M30 = 0.82 and M65 = 0.80). FAS is a member of the tumor necrosis factor receptor superfamily, and studies have demonstrated that it plays an important role in NAFLD. In recent years, the combination of CK18 and FAS has attracted more and more attention in the diagnosis of NASH, and the combination of M30 and FAS levels can improve the diagnostic accuracy of NASH (AUROC of 0.79 - 0.93)[5].

According to US Food and Drug Administration (FDA), CK18 is one of the most promising biomarkers and the most extensively studied biomarker for NASH. But there are still some limitations, such as insufficient accuracy and lack of extensive experimental studies[6-7].

### NASH fibrosis-specific markers

NASH liver fibrosis is characterized by abnormal deposition of extracellular matrix in liver tissue caused by its extensive proliferation, which finally leads to abnormalities in liver structure and function[8]. The noninvasive serological markers for liver fibrosis can be divided into two types: direct and indirect markers. Direct serologic markers are the products of the update of extracellular

matrix during liver fibrosis, including extracellular matrix components, degradation products, enzymes and cytokines involved, such as hyaluronic acid (HA), laminin (LN), procollagen type III (Pc III), and collagen type IV (C IV). Indirect serological markers reflect liver function or inflammation, such as ALT, AST, gamma-glutamyl transpeptidase (GGT), *etc.* The two types are usually used in combination to form a scoring system or a mathematical model to grade fibrosis in association with the age and body fat ratio of patients[5]. Novel systems used for diagnosis of NASH-specific liver fibrosis include enhanced liver fibrosis (ELF), FibroTest/FibroSURE, Fibro Meter, Hepascore and NIS4.

Hyaluronic acid (HA) is a major component of the extracellular matrix. Using serum HA level alone as a criterion, the AUROC for diagnosis of fibrosis ($\geqslant$ F2) is only 0.87, and that for diagnosis of cirrhosis is 0.92. In another study, the AUROC for diagnosing fibrosis at stage F3 – F4 is 0.82[9].

Serum procollagen type III amino-terminal peptide (PIIINP) is an amino-terminal peptide produced by the cleavage of procollagen type III before it is secreted into extracellular space. PIIINP alone is not a good marker for diagnosing liver fibrosis of NAFLD or alcoholic liver diseases[5].

Tissue inhibitor of metalloproteinases 1 (TIMP1), which regulates matrix metalloproteinases, makes up the extracellular matrix and promotes wound healing, reflects the changes in tissue matrix remodeling during liver fibrosis and fibrinolysis[7]. TIMP1 has excellent diagnostic accuracy, with an AUROC of 0.97 for the diagnosis in obese NASH patients[10].

Enhanced liver fibrosis (ELF) score is a biomarker for diagnosis of advanced fibrosis based on the fibrosis-specific markers mentioned above: PIIINP, HA and TIMP1[11]. ELF can accurately predict advanced fibrosis in adult and pediatric patients with NAFLD (AUROC of 0.93 and 0.99, respectively)[12].

Fibro Test/FibroSURE involves five indicators: total bilirubin, GGT, α2-macroglobulin (α2-MG), apolipoprotein A1 and globulin, adjusted for age and gender. The AUC of FibroTest is only 0.77 for testing liver fibrosis, but 0.92 for cirrhosis[13].

Fibrometer involves seven indices: platelets, prothrombin index (PI), AST, α2-MG, serum creatinine, HA and age, based on liver disease stages. In a study including 95 patients with NAFLD, Fibrometer index reaches an AUC of 0.96 for the diagnosis of moderate to severe liver fibrosis (F2 – F4). This method is more accurate for the diagnosis of alcoholic liver fibrosis than APRI and FibroTest. To meet technological and clinical demands, Loong *et al.* [14] proposed a new system which combines Fibrometer with vibration-controlled transient elastography algorithm to detect Chinese patients with NAFLD, and the results showed that it has high diagnostic accuracy, with AUC of 0.855 for F2-F4 and 0.901 for F3-F4 fibrosis, respectively, and the positive predictive value of the new system is also higher than that of TE alone. So, the combination of Fibrometer and transient elastography not only has an important role in diagnosing F2-4 and F3-4 fibrosis, but also improves the positive predictive value.

Hepascore combines HA, α2-MG, total bilirubin and GGT with gender and age. The study of Adams *et al.* [15] showed that it provides the highest specificity and positive predictive value for predicting advanced fibrosis, and the AUROC value reaches 0.90 for detecting cirrhosis.

The latest NIS4 algorithm comprises microRNA-34a-5p, α2-MG, YKL-40 and glycated hemoglobin. Patients with a NIS4 value $<0.36$ are classified as not having at-risk NASH, with a sensitivity of 81.5%, a specificity of 63.0%, and a negative predictive value of 77.9%, while those with a NIS4 value $>0.63$ are classified as having at-risk NASH with a specificity 87.1%, a sensitivity 50.7%, and a positive predictive value 79.2%. The most significant advantage of the algorithm is that its performance is not affected by age, gender, BMI or transaminase concentration[16].

Although these NASH fibrosis-specific markers are accurate predictors of advanced liver fibrosis and cirrhosis, the high cost limits their use in clinical practice. In addition, age of patients and other factors may affect the accuracy of these methods[17]. Recent reports have suggested that combining biomarkers with elastography, such as the combination of vibration-controlled transient elastography (VCTE) with FibroMeter (AUROC: 0.86) and the combination of FibroTest with TE, can improve the diagnostic accuracy of liver fibrosis[18].

## Scoring Systems for NASH

### PRO-C3-based ADAPT score

**Reliability of PRO-C3 for diagnosing fibrosis** PRO-C3 is a serological biomarker, which can measure the formation of type III collagen, a major scar-associated collagen that is deposited during fibrogenesis by activated myofibroblasts. The assay measures the type III collagen formation epitope generated by ADAM-TS2 during release of the N-terminal pro-peptide, in contrast to the classical PIIINP (procollagen III amino terminal pro-peptide) that measures an internal fragment of PIIINP related to both formation and degradation of type III collagen. Thus, PRO-C3, a biomarker that correlates with the stage and kinetics of liver fibrosis, can predict the progression and clinical outcome of this disease, and can be used as a pharmacodynamic biomarker for metabolic and cholestatic liver disease[19].

**ADAPT algorithm based on PRO-C3** By conducting logistic regression analysis on various clinical variables of more than 280 patients with non-alcoholic fatty liver disease (NAFLD), the ADAPT algorithm is established as[3].

$$\mathrm{ADAPT} = \exp\left(\log_{10}\left(\frac{\mathrm{Age}\times\mathrm{PRO}-\mathrm{C3}}{\sqrt{\mathrm{Platelets}}}\right)\right) + \mathrm{Diabetes}$$

The variables included in the model are age, presence of diabetes, platelet count and the marker PRO-C3. The diagnostic capability of the ADAPT score is assessed via AUROC and is higher than that of PRO-C3 alone, yielding an AUROC of 0.86 (95% confidence interval of 0.79 – 0.91).

**Accuracy of ADPAT algorithm** A separate cohort ($n=281$)

comprised of patients from four centers in Asia Pacific and Europe confirmed the ability of ADPAT to identify patients with advanced fibrosis[3]. FIB-4 and APRI showed reasonable performance at identifying patients without advanced liver fibrosis, 68% ($n=147$) and 67% ($n=145$) patients were correctly classified, respectively. However, these scores performed poorly at identifying patients with advanced liver fibrosis, NAFLD Fibrosis Score and FIB-4 correctly identified 51% ($n=33$) and 46% ($n=30$) patients, respectively. In contrast, by applying ADAPT score, 73% ($n=158$) of F0-2 patients and 92% ($n=60$) of F3-4 patients were correctly classified.

In univariate logistic regression analysis, all the four non-invasive scores were associated with significant and progressive fibrosis and NASH. In multivariate logistic regression analysis, ADAPT was independently associated with significant fibrosis and NASH with ORs of 1.738 and 2.186, respectively, and had the highest accuracy in the diagnosis of F3 – F4 fibrosis[20].

ADAPT score which is based on PRO-C3 and routine clinical parameters can identify patients with advanced fibrosis in NASH patients, and thus can be adopted for risk stratification and for the clinical management of patients with NAFLD. However, further independent studies will be required to determine whether patient stratification using ADAPT followed by measurement of liver stiffness can replace the need for liver biopsy as a diagnostic standard in NAFLD.

**Diagnostic effectiveness of APRI score, FIB-4 score, BARD score, and NAFLD fibrosis score**

Liver fibrosis is an important pathological feature of NASH, which is mainly caused by the proliferation of hepatic stellate cells (HSC) when the liver is damaged[1]. AST/ALT ratio, APRI (aspartate aminotransferase-to-platelet ratio index) score, FIB-4 (fibrosis-4) score, BARD (body Mass Index, AST/ALT ratio, diabetes) score, and NAFLD fibrosis score (NFS) are common predictors for liver fibrosis. APRI score includes AST and platelet count. FIB-4 score includes age, AST, ALT and platelet count. BARD score is comprised of BMI, diabetes and AST/ALT ratio. NAFLD fibrosis score consists of age, BMI, albumin, AST/ALT ratio, hyperglycemia and platelet count[21]. The diagnostic effectiveness of the four scores is analyzed here.

FIB-4 is more accurate in predicting stages 0 and 1-4 liver fibrosis in NASH. Thirteen studies involving 6 557 adult patients were included in the qualitative assessment by Ismaie A *et al.* [22], out of which, six studies were included in the quantitative assessment. Prediction of NASH was evaluated better using NFS (AUC of 0.687) and FIB-4 (AUC of 0.729). Fibrosis stages 0 vs. 1 – 4 was diagnosed better using NFS (AUC of 0.718) and FIB-4 (AUC of 0.723). Advanced fibrosis was assessed better by BARD (AUC of 0.673), APRI (AUC of 0.762), NFS (AUC of 0.787) and FIB-4 (AUC of 0.821). The results indicated that FIB-4 predicts NASH and quantified liver fibrosis, stages 0 vs. 1 – 4 more precisely compared to NFS, APRI, and BARD.

APRI and hyaluronic acid have superior predictive power for hepatic cirrhosis (F4) than for significant fibrosis (F2-F3). In another study, the pooled estimate for sensitivities and specificities of APRI and hyaluronic acid to diagnose F4 are (84% and 82%) and (83% and 89%) respectively. In the treatment of patients with hepatitis C at early stages, APRI had better diagnostic performance in diagnosing liver cirrhosis with 93.8% sensitivity and 72.4% specificity (AUC 0.908, 95% CI 0.851 – 0.965, $p<0.001$) compared to its accuracy in diagnosing significant hepatic fibrosis[23].

The combined use of BARD and BAAT results in high sensitivity and therefore can be used for the initial screening of NAFLD, while higher specificity is achieved when NFS or APRI is used alone, and thus they can be used for differential diagnosis of NAFLD[24].

## Diagnostic Imaging Techniques for NASH

Ultrasound, magnetic resonance elastography (MRE) and TE are most widely used imaging techniques to assess the progression of NASH. Here, we present them in diagnosis of steatosis and fibrosis, respectively.

**Diagnosis of steatosis**

Conventional techniques to assess steatosis include ultrasonography, computed tomography (CT) and MR spectroscopy and MRI. Among them, ultrasonography, the most commonly used and simplest method, allows grading of hepatic steatosis, and has a sensitivity of 85% (80% – 89%) and a specificity of 93% (87% – 97%) for the diagnosis of moderate to severe fatty liver disease, but a poor sensitivity of only 65% and a poor specificity of only 81% for detecting micro steatosis and, respectively[25]. Therefore, it is unable to distinguish between NAFL, NASH and cirrhosis. A new quantitative ultrasound (QUS) score was developed to identify NASH, and showed good discriminatory capacity and calibration for identifying NASH both in the training set (AUROC: 0.798, 95% CI 0.731 – 0.865; Hosmer – Lemeshow test, $P=0.755$) and in the validation set (AUROC: 0.816, 95% CI 0.725 – 0.906; Hosmer – Lemeshow test, $P=0.397$), providing a novel, non-invasive and practical new idea for identifying NASH[26]. Conventional CT and MRI do have some value for semi-quantitative quantification of hepatic steatosis. However, CT is unable to accurately diagnose mild steatosis regardless of the potential hazards of exposure to ionizing radiation.

Magnetic resonance imaging derived proton density fat fraction (MRI-PDFF) has been demonstrated to be superior to liver biopsy in several studies[27-30]. It is more accurate than CAP for the quantification of liver steatosis of all grades in NAFLD patients (with an AUROC 0.99)[31]. But its disadvantages are obvious such as being expensive, and probably inaccurate when affected by acute hepatic inflammation or iron burden[31], and thus MRI-PDF is unable to assess liver inflammation, NASH regression or fibrosis improvement[32]. In conclusion, MRI-PDFF is emerging as a diagnostic method for the assessment of hepatic steatosis.

**Diagnosis of fibrosis**

The most accurate noninvasive methods for assessing liver

fibrosis are those based on liver elastography, including vibration-controlled transient elastography (VCTE), MRE, shear-wave elastography, and acoustic radiation force impulse imaging.

Controlled attenuation parameter (CAP) and liver stiffness measurement (LSM) by VCTE based FibroScan assess liver steatosis and fibrosis, respectively. CAP cannot accurately grade steatosis, and LSM has low accuracy and a high technical failure rate for detecting mild fibrosis[33].

MRE, a phase contrast-based MRI technique, is more accurate than TE for the assessment of liver fibrosis[34], but its use is limited by high cost, examination time and availability, *etc.* TE has a high accuracy for the detection of advanced fibrosis (for F3, sensitivity 85%, specificity 82%; and for F4, sensitivity 92%, specificity 92%) but a lower accuracy for significant fibrosis (for F2, sensitivity 79%, specificity 75%)[35]. Therefore, TE is often used to detect advanced fibrosis. However, neither of the two approaches can distinguish between NASH and simple steatosis[36]. Shear-wave elastography (SWE) is noninvasive, painless, rapid, simple and quantitative. Studies have found no difference in accuracy between 2D SWE and MRE in diagnosing significant and advanced fibrosis[37]. SWE has shown promising potential in fibrosis diagnosis for its numerous advantages. For example, it can selectively avoid the major vascular structures in the liver, its sample size is adjustable, and its application is not limited by other factors such as obesity and pregnancy, all of which make it a promising for application[38].

Acoustic radiation force impulse imaging (ARFI) is an ultrasound elastography technique that has been widely used in the diagnosis of the extent of liver fibrosis, but has not been extensively evaluated for the diagnosis of NASH.

In conclusion, MRE is the best noninvasive biomarker for quantification of liver fibrosis, with a low failure rate in obese patients, whereas VCTE and all ultrasound-based modalities may have unreliable readings[33]. Future studies should focus more on whether there is a specific BMI threshold, below which VCTE or ultrasound techniques should be used first, and above which MRE should be used first.

Meanwhile, the combinations of biomarkers with diagnostic imaging techniques have made some achievements in fibrosis diagnosis. With a three-step strategy in which FIB-4 is combined with 2D-SWE-SSI and VCTE, a study analysis on a large cohort of MAFLD patients proved that the percentage of unclassified patients is exceptionally low ($<5\%$), with a diagnostic accuracy greater than 80%. Another study adopted FibroScan-AST (FAST) score to identify patients with NAFLD, patients with NASH, patients with intermediate liver fibrosis ($\geq$F2) and patients with high NAFLD activity scores (NAS $\geq$4), and acquired an AUROC of 0.85 (0.83 – 0.87), a sensitivity of 0.89 and a specificity of 0.92, respectively.

## Summary

Although liver biopsy is currently the gold standard for diagnosing nonalcoholic steatohepatitis, it still has some drawbacks that cannot be ignored, such as invasiveness and sampling error. It also crates some confusion to the differential diagnosis of nonalcoholic steatohepatitis and liver fibrosis, which will affect the subsequent clinical treatment and prognosis. In this paper, we introduce and analyze the advantages and disadvantages of existing non-invasive diagnostic methods for nonalcoholic steatohepatitis, such as the biomarkers of hepatocellular fibrosis, scoring systems composed of different physiological indices, and medical imaging techniques. However, the best method for differential diagnosis of nonalcoholic steatohepatitis is still inconclusive. This paper is expected to provide useful references for the development of new diagnostic criteria for nonalcoholic steatohepatitis.

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