**Title Page**

**Title**

Development and validation of a novel non-invasive test for diagnosing fibrotic NASH in patients with biopsy-proven NAFLD

**Short Title:** Non-invasive diagnosis of fibrotic NASH

**Authors’ name**

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**Conflict of Interest Statement**

All authors: nothing to declare.

**Abbreviations**

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; MetS, metabolic syndrome; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, γ-glutamyl transpeptidase; HOMA-IR, homeostasis model assessment-insulin resistance; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; TC, total cholesterol; NAS, NAFLD activity score; FIB-4, Fibrosis-4 index; NFS, NAFLD fibrosis score; AUROC, area under the receiver operator characteristic curve; OR, odds ratio; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value

**Ethical approval:** Ethical approval for the study was obtained from the ethics committee of the First Affiliated Hospital of Wenzhou Medical University and University of Malaya. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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included in the study.

**ABSTRACT**

**Introduction:** There is an immediate need for non-invasive accurate tests for diagnosing liver fibrosis in patients with nonalcoholic steatohepatitis (NASH). Previously, it has been suggested that MACK-3 (a formula that combines HOMA-insulin resistance with serum AST and cytokeratin [CK]18-M30 levels) accurately identifies patients with fibrotic NASH. Our aim was to assess the performance of MACK-3 and develop a novel, non-invasive algorithm for diagnosing fibrotic NASH.

**Methods:** 636 adults with biopsy-proven nonalcoholic fatty liver disease (NAFLD) from two independent Asian cohorts were enrolled in our study. Liver stiffness measurement (LSM) was assessed by vibration-controlled transient elastography (Fibroscan®). Fibrotic NASH was defined as NASH with a NAFLD activity score (NAS)≥4 and F≥2 fibrosis.

**Results:** Metabolic syndrome (MetS), platelet count and MACK-3 were independent predictors of fibrotic NASH. Based on their regression coefficients, we developed a novel nomogram showing a good discriminatory ability [AUROC: 0.79, 95%CI 0.75-0.83] and a high negative predictive value (NPV: 94.7%) to rule out fibrotic NASH. In the validation set, this nomogram had a higher AUROC (0.81, 95%CI 0.74-0.87) than that of MACK-3 (AUROC: 0.75, 95%CI 0.68-0.82; *p*<0.05) with a NPV of 93.2%. The sequential combination of this nomogram with LSM data avoided the need for liver biopsy in 56.9% of patients.

**Conclusions:** Our novel nomogram (combining MACK-3, platelet count and MetS) shows promising utility for diagnosing fibrotic NASH. The sequential combination of this nomogram and vibration-controlled transient elastography limits indeterminate results and reduces the number of unnecessary liver biopsies.

**KEYWORDS:** NAFLD, clinical; NASH; Diagnostic tests; Liver fibrosis; Liver biopsy

**Introduction**

Nonalcoholic fatty liver disease (NAFLD) is a multifactorial metabolic liver condition that affects up to 25% of the global adult population.[1](#_ENREF_1) The characteristic feature of NAFLD is an excessive fat deposition in hepatocytes, in the absence of significant alcohol consumption and other known causes of chronic liver disease.[2](#_ENREF_2) The histopathologic spectrum of NAFLD comprises simple steatosis (NAFL), nonalcoholic steatohepatitis (NASH), advanced fibrosis and cirrhosis.

Recently, the phase 3 REGENERATE trial has reported preliminary positive results with the use of obeticholic acid (i.e., an agonist of the farnesoid X receptor) on the histologic features of NASH in patients with stage 2 or 3 liver fibrosis.[3](#_ENREF_3) The RESOLVE-IT trial is also an ongoing phase 3 randomized controlled trial testing the possible benefit of elafibranor (i.e., an agonist of the peroxisome proliferator-activated receptor-α and peroxisome proliferator-activated receptor-δ) on NASH resolution without worsening of fibrosis.[4](#_ENREF_4) The need to treat NASH patients with varying amounts of fibrosis creates a demand for accurately identifying patients with fibrotic NASH, who may derive the most benefit from pharmacologic treatment.[3](#_ENREF_3)

Liver biopsy remains the reference standard for assessing NASH and staging liver fibrosis. However, liver biopsy is invasive and expensive.[5](#_ENREF_5) In addition, the results of liver biopsy only provide a nonlinear and semiquantitative assessment of liver disease.[6](#_ENREF_6) This may limit the precision and granularity of data, particularly in the context of subtle changes in therapeutic responses.[7](#_ENREF_7) Therefore, a number of non-invasive tests (NITs) for liver fibrosis, including assessment of biomarker panels and imaging techniques have been developed to assess the severity of liver disease.[8-10](#_ENREF_8) In a study involving 846 patients with biopsy-proven NAFLD from three European centers (Angers, Nice, Antwerp), Boursier *et al*. recently developed the MACK-3 model (i.e., a formula including HOMA-estimated insulin resistance score, serum aspartate aminotransferase [AST] and cytokeratin-18 fragment [CK18-M30] levels) with the aim of screening patients for fibrotic NASH.[11](#_ENREF_11) The diagnostic performance of MACK-3 has been externally validated in a sample of 196 Malaysian patients with NAFLD by Chuah *et al*.,[12](#_ENREF_12) who reported the model’s strength in identifying patients with active NASH instead of fibrotic NASH. Moreover, the performance of MACK-3 for the diagnosis of fibrotic NASH was not superior to other widely used NITs, such as the NAFLD fibrosis score (NFS) or the fibrosis-4 (FIB-4) index.

Thus, the major aims of our study were to: (1) assess the accuracy of MACK-3 in diagnosing fibrotic NASH; (2) develop and validate a new non-invasive diagnostic tool to diagnosis fibrotic NASH; and (3) assess whether the sequential and combined use of this newly developed diagnostic tool (that includes also a measurement of liver stiffness obtained by vibration-controlled transient elastography) increases the number of correctly avoided liver biopsies.

**Methods**

***Study population and design***

We studied a sample of 642 adults with suspected NAFLD who were consecutively recruited at the First Affiliated Hospital of Wenzhou Medical University in Wenzhou (China) from December 2016 to December 2018. We then excluded 202 patients for the following main reasons: (1) significant alcohol intake (≥ 140 g/week in men or ≥ 70 g/week in women); (2) use of steatosis-inducing drugs and presence of viral hepatitis, autoimmune hepatitis or other known chronic liver diseases; (3) incomplete clinical and biochemical data. As result of this exclusion, we included 440 patients with biopsy-confirmed NAFLD in the Wenzhou cohort.

We also included another group of 196 adults with biopsy-confirmed NAFLD who were recruited to our previously published study.[12](#_ENREF_12) These subjects were enrolled at the University of Malaya Medical Centre in Kuala Lumpur (Malaysia) between 2012 and 2015. The inclusion and exclusion criteria were consistent with those of the Wenzhou cohort.

The study protocol was approved by the local ethics committees of Wenzhou Medical University First Affiliated Hospital and University of Malaya, respectively, as reported previously.[12-14](#_ENREF_12) Consent has been obtained from each patient or subject after full explanation of the purpose and nature of all procedures used.

***Metabolic syndrome definition***

Metabolic syndrome (MetS) was defined as having at least three of the following metabolic risk factors: central obesity (i.e., waist circumference ≥90 cm in men and ≥80 cm in women); increased blood pressure (systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg or use of anti-hypertensive drugs); elevated fasting glucose (≥5.6 mmol/L or use of anti-hyperglycemic agents); high triglycerides (>1.7 mmol/L or use of lipid-lowering drugs); and low HDL-cholesterol levels (<1.03 mmol/L in men and <1.29 mmol/L in women, or use of lipid-lowering drugs).[15](#_ENREF_15)

***Clinical and laboratory data***

Anthropometric and routine laboratory data were obtained from all participants within 24 hours of liver biopsy. All blood samples were taken in fasting conditions. Methodology for measurement of plasma levels of cytokeratin-18 fragments (CK-18 neoepitope M30) has been reported previously.[12](#_ENREF_12), [16](#_ENREF_16) The homeostasis model assessment-insulin resistance (HOMA-IR), MACK-3, NFS and FIB-4 scores were calculated from published formulas.[11](#_ENREF_11), [17](#_ENREF_17), [18](#_ENREF_18)

***Liver histology***

Liver histology assessments were undertaken by experienced liver pathologists and consensus scores determined according to the NASH-Clinical Research Network Scoring System.[19](#_ENREF_19) Liver pathologists were blinded to the patients’ clinical and biochemical details. A diagnosis of NASH required at least one point each for the presence of steatosis, ballooning and lobular inflammation. [11](#_ENREF_11), [20](#_ENREF_20) In accord with previously published studies, active NASH was defined as the presence of NASH and a NAFLD activity score (NAS) ≥4, whereas fibrotic NASH was defined as the presence of active NASH and significant fibrosis (F ≥2).[11](#_ENREF_11), [12](#_ENREF_12)

***Liver stiffness measurement***

LSM values were measured by two experienced operators, using vibration-controlled transient elastography technology (Fibroscan®; Echosens, Paris, France), according to the manufacturer’s recommendations.[21](#_ENREF_21) All patients underwent FibroScan® examination within two weeks of liver biopsy and they were asked to fast at least three hours before the examination. In brief, each LSM measurement was considered adequate when including at least 10 valid measurements for each patient, with a success rate >60% and variability of measurements (IQR/M) <30% of the median.

***Statistical analysis***

Continuous variables were expressed as means ± SD or medians with interquartile ranges (IQRs), according whether the distribution was normal or skewed. Categorical variables were expressed as percentages. The unpaired Student *t*-test (for variables normally distributed), the Mann-Whitney U-test (for variables non-normally distributed) and the Chi-squared test (for categorical variables) were used to investigate the differences between the groups. The performance of the NITs was evaluated by calculating the area under the receiver operating characteristic curve (AUROC) and compared by the DeLong test. The model calibration was assessed by the Hosmer-Lemeshow goodness of fit test.

For the development of our nomogram, the study population (n = 636) was randomly assigned at a 3:1 ratio (training set: 472 patients; validation set: 164 patients) using a split-sample method by an experienced statistician. In the training set, variables selected from univariable logistic regression analysis (*p*<0.10), or considered clinically significant, were introduced as covariates in a multivariable logistic regression model. Variables that were statistically significant (*p*<0.05) in this multivariate regression model were then used to develop the nomogram. Odds ratios (OR) are reported with a 95% confidence interval (CI). The accuracy of this novel diagnostic model was subsequently evaluated in the validation set. Finally, we explored whether a two-step approach combining the nomogram, or MACK-3, with the LSM reduced the necessity for liver biopsies. The sensitivity, specificity, NPV, positive predictive value (PPV), percentage of misclassifications, and indeterminate or discordant results of all tests were calculated at each cut-off. The percentage of patients who correctly avoid liver biopsies was determined by dividing the number of patients without fibrotic NASH correctly identified by NITs by the total number of patients.

The diagnostic cut-offs for LSM and the nomogram, corresponding to the 95% negative predictive value (NPV) and 90% specificity thresholds for fibrotic NASH, were firstly calculated in the training set. Statistical analyses were performed using R (version 3.3.1 The R Foundation) and MedCalc (version 15.2.2 Ostend, Belgium). Statistical significance was set at *p* <0.05.

**Results**

***Baseline characteristics of patients***

Based on the inclusion and exclusion criteria of the study, a total of 636 adult individuals with biopsy-confirmed NAFLD from two tertiary hepatology centers were included in the study (Supplementary Table 1). The mean age of these patients was 44 years and 67.0% of them were male. The mean values of waist circumference and BMI were 93.2 cm and 27.6 kg/m2, respectively. 232 patients (36.5%) had established type 2 diabetes and 389 (61.2%) patients had MetS. The median value of the NAS score was 4 (IQR 3-5). NASH was diagnosed in 445 patients (70.0%) and fibrotic NASH in 103 patients (16.2%) (Table 1).

***Development of a novel nomogram to rule out fibrotic NASH***

In the training set (n=472 patients), the multivariable regression analysis showed that MetS, platelet count and MACK-3 were significantly associated with the presence of fibrotic NASH (Supplementary Table 2). Prediction of fibrotic NASH using our nomogram was developed from the regression coefficients (Figure 1). For example, a patient whose MACK-3 was 0.10, platelet count was 250 ×109/L, and without obesity, the total points scored was 58, the probability of fibrotic NASH was no more than 1%. To accurately calculate the nomogram score, we provided the following formula: MACK-3 \* 78 + [400-platelet count (×109/L)] \* 0.157] + MetS (patients without obesity =25, with obesity but without MetS =79, with obesity and MetS =100) +2. (N.B. a simple and user-friendly calculator to estimate the individual’s risk of fibrotic NASH can be accessed at the following web site <https://wzmu.shinyapps.io/DynNomapp/>).

The discriminatory ability of our nomogram [AUROC 0.79, 95%CI 0.75-0.83 in the training set (n=472 patients), and 0.81, 95 %CI 0.74-0.87 in the validation set (n=164 patients)] was significantly superior to MACK-3, NFS and FIB-4 scores (Figure. 2). The nomogram also showed good calibration both in the training and validation sets (Hosmer-Lemeshow test: *p* values = 0.58 and 0.78, respectively). With the specific aim of identifying (by ROC curves) the most accurate nomogram cut-off values for ruling out fibrotic NASH (training set: NPV: 94.7%; specificity: 93.2%), we used two cut-off values of ≤ 137 and ≥ 180, respectively. In the validation set, the cut-off value ≤ 137 had an NPV of 93.2% to rule out fibrotic NASH, whereas the cut-off value ≥ 180 had a specificity of 90.4% to rule in fibrotic NASH.

***Practical algorithm combined with LSM***

In our study, there were 357 patients who underwent FibroScan® examination (164 patients from the Wenzhou cohort and 193 patients from the Kuala Lumpur cohort). We calculated two cut-offs for LSM corresponding to the 95% NPV and 90% specificity thresholds. Finally, we defined the optimal cut-off values of LSM at 6.2 kPa to exclude fibrotic NASH and at 12.0 kPa to confirm fibrotic NASH [in the whole study population: sensitivity 92.2% (59/64); specificity 94.9% (279/294); NPV: 97.3% (145/149); PPV: 65.1% (28/43)].

To further reduce the percentage of indeterminate results and to improve the number of liver biopsies correctly avoided, we developed an algorithm combining the nomogram and LSM measurements (Figure 3A). Using this combined nomogram-LSM algorithm, the NPV remained very high (95%) and the number of liver biopsy correctly avoided, compared to nomogram or LSM alone, increased from 56.7% (93/164) and 53.7% (88/164), respectively, to 68.3% (112/164) in the Wenzhou cohort (*p*=0.030 and 0.007, respectively), and from 36.8% (71/193) and 29.5% (57/193), respectively, to 47.2% (91/193) in the Kuala Lumpur cohort (*p*=0.039 and <0.001, respectively) . This combined nomogram-LSM algorithm also improved the PPV by at least 25% and reduced the misclassification rates and indeterminate results in both cohorts (Table 2). Although five patients were incorrectly diagnosed as having fibrotic NASH according to the combined nomogram-LSM algorithm, it is important to note that all of these patients had NASH (NAS ≥4) with mild fibrosis (F=1) on histology.

We also tried to develop the MACK-3 score utility further, by combining MACK3 with LSM measurements in another algorithm (Figure 3B). This algorithm (combining MACK-3 and LSM) improved the specificity, PPV, and the number of liver biopsies correctly avoided, and also reduced the misclassification rate. However, this algorithm increased the numbers of indeterminate results. Moreover, compared to the nomogram-LSM algorithm, the MACK3-LSM algorithm produced a higher percentage of indeterminate results (32.9% vs. 25.6% in the Wenzhou cohort; 47.2% vs. 42.5% in the Kuala Lumpur cohort), and lower number of liver biopsies correctly avoided [101/164 (61.6%) vs. 112/164 (68.3%) in the Wenzhou cohort; 72/193 (37.3%) vs. 91/193 (47.2%) in the Kuala Lumpur cohort].

**Discussion**

In this large cross-sectional study, we have used a combined cohort of 636 adult individuals with biopsy-proven NAFLD to develop and validate a new non-invasive algorithm for ruling out fibrotic NASH. Our algorithm had better discriminatory ability for diagnosing fibrotic NASH in both the training and validation sets than other widely used NITs. In addition, to our knowledge, ours is also the largest study that has validated the use of the MACK-3 model that was recently developed by Boursier et al..[11](#_ENREF_11)

We measured MACK-3’s performance in diagnosing fibrotic NASH and found that it was moderately accurate in our cohort, although this finding could be affected by differences in fibrotic NASH prevalence between the studied populations. Through multivariable logistic regression analyses, we have demonstrated that MetS, platelet count and MACK-3 were the three strongest predictors of fibrotic NASH. Platelet count is known to closely correlate with advanced liver fibrosis and is considered to be an important variable in several NITs, including the FIB-4 and NFS scores.[17](#_ENREF_17), [18](#_ENREF_18), [22](#_ENREF_22) Obesity and MetS are well known to be strongly associated with NASH and fibrosis.[23](#_ENREF_23) By combining platelet count, MetS and MACK-3 score, we then developed a new nomogram to rule out fibrotic NASH. By incorporating additional biomarkers associated with NASH and especially liver fibrosis, our newly developed nomogram resulted in a significantly better diagnostic performance compared to MACK-3, FIB-4 or NFS scores, respectively.

The nomogram, MACK-3 and LSM all showed good capability of ruling out fibrotic NASH, due to their high NPVs. However, their ability to rule in the diagnosis of fibrotic NASH was suboptimal, due to their low PPVs. In addition, all available NITs tend to have a “grey” zone of intermediate results due to the usual two-cutoff thresholds.[24](#_ENREF_24) To try to circumvent this problem, a two-step approach has previously been suggested combining LSM with NITs, in order to increase the overall PPV and thereby effectively decrease the number of patients in the “grey” zone.[24](#_ENREF_24), [25](#_ENREF_25) Similar to this approach, we constructed a sequential algorithm that firstly evaluated the probability of fibrotic NASH by using the new nomogram and subsequently added the LSM data obtained with FibroScan®. Interestingly, for this sequential approach (by combining nomogram and LSM) we observed a better performance than employing either strategy alone. The two-step sequential approach of using either the nomogram followed by LSM (nomogram-LSM algorithm), or MACK-3 followed by LSM (MACK-3-LSM algorithm); both diagnostic approaches showed a high NPV (95%) for ruling out fibrotic NASH. In addition, both approaches also produced a low number of patients requiring liver biopsy and low rates of misclassification.

The combination of our nomogram with LSM avoided liver biopsies in an additional 68.3% of subjects in the Wenzhou cohort. Similar results were also confirmed in the Kuala Lumpur cohort where the combined nomogram-LSM algorithm would have avoided liver biopsy in 47.2% of subjects. Moreover, and most importantly, the proportion of avoided liver biopsies by using the combined nomogram-LSM algorithm was significantly higher than that of the combined MACK3-LSM algorithm.

Previous studies reported that patients with NAFLD who have serum AST levels <35 UI/L and who do not have MetS are considered very unlikely to have fibrotic NASH, and that liver biopsy could be avoided in this subgroup of patients.[11](#_ENREF_11) Similar findings are present in our study, in that the prevalence of fibrotic NASH was only 2% amongst these patients.

There are several limitations to our study. Firstly, the baseline characteristics of patients enrolled by the two hepatology centers are different (Supplementary Table 1). This heterogeneity may contribute to the variability of NITs’ diagnostic performances when comparing results in one cohort versus the other. Secondly, our patients with NAFLD are of Asian ethnicity and therefore may not accurately represent the characteristics of other ethnic groups of patients. Further studies are needed to determine the utility of our non-invasive algorithm in non-Asian individuals with NAFLD. Thirdly, the results of MACK-3 might be also affected by pre-existing diabetes. Therefore, we stratified our analyses either by diabetes status or by the use of certain antihyperglycemic agents (insulin or insulin sensitizers). In these subgroup analyses, we found that there were no significant differences in the diagnostic performances of both MACK-3 and our newly developed nomogram for diagnosing fibrotic NASH (Supplementary Table 3). Moreover, to limit the influence of supra-physiological results of hyperinsulinemia induced by insulin therapy, we capped the HOMA-IR at 10 while calculating the MACK-3 as previously undertaken by Boursier et al..[11](#_ENREF_11)

In conclusion, our novel nomogram (combining MACK-3, platelet count and presence of MetS) shows promising utility for diagnosing fibrotic NASH. In addition, the sequential two-step approach of combining our nomogram with LSM data obtained with vibration-controlled transient elastography (FibroScan®) significantly reduces the number of both indeterminate results and unnecessary liver biopsies.

**REFERENCE**

[1] Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology (Baltimore, Md)*. 2016; 64: 73-84.

[2] Brunt EM, Wong VW, Nobili V*, et al.* Nonalcoholic fatty liver disease. *Nature reviews Disease primers*. 2015; 1: 15080.

[3] Alkhouri N, Lawitz E, Noureddin M. Looking Into the Crystal Ball: Predicting the Future Challenges of Fibrotic NASH Treatment. *Hepatology communications*. 2019; 3: 605-13.

[4] Ratziu V, Harrison SA, Francque S*, et al.* Elafibranor, an Agonist of the Peroxisome Proliferator-Activated Receptor-α and -δ, Induces Resolution of Nonalcoholic Steatohepatitis Without Fibrosis Worsening. *Gastroenterology*. 2016; 150.

[5] Rockey DC, Caldwell SH, Goodman ZD, Nelson RC, Smith AD. Liver biopsy. *Hepatology (Baltimore, Md)*. 2009; 49: 1017-44.

[6] Brunt EM. Nonalcoholic fatty liver disease and the ongoing role of liver biopsy evaluation. *Hepatology communications*. 2017; 1: 370-8.

[7] Liu F, Goh GB, Tiniakos D*, et al.* qFIBS: A Novel Automated Technique for Quantitative Evaluation of Fibrosis, Inflammation, Ballooning, and Steatosis in Patients With Nonalcoholic Steatohepatitis. *Hepatology (Baltimore, Md)*. 2019; doi: 10.1002/hep.30986.

[8] Castera L, Friedrich-Rust M, Loomba R. Noninvasive Assessment of Liver Disease in Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology*. 2019; 156: 1264-81.e4.

[9] Vilar-Gomez E, Chalasani N. Non-invasive assessment of non-alcoholic fatty liver disease: Clinical prediction rules and blood-based biomarkers. *Journal of hepatology*. 2018; 68: 305-15.

[10] Zhou JH, Cai JJ, She ZG, Li HL. Noninvasive evaluation of nonalcoholic fatty liver disease: Current evidence and practice. *World journal of gastroenterology*. 2019; 25: 1307-26.

[11] Boursier J, Anty R, Vonghia L*, et al.* Screening for therapeutic trials and treatment indication in clinical practice: MACK-3, a new blood test for the diagnosis of fibrotic NASH. *Alimentary pharmacology & therapeutics*. 2018; 47: 1387-96.

[12] Chuah KH, Wan Yusoff WNI, Sthaneshwar P, Nik Mustapha NR, Mahadeva S, Chan WK. MACK-3 (combination of hoMa, Ast and CK18): A promising novel biomarker for fibrotic non-alcoholic steatohepatitis. *Liver international : official journal of the International Association for the Study of the Liver*. 2019; 39: 1315-24.

[13] Zhou YJ, Ye FZ, Li YY*, et al.* Individualized risk prediction of significant fibrosis in non-alcoholic fatty liver disease using a novel nomogram. *United European Gastroenterology Journal*. 2019; 7: 1124-34.

[14] Sun D-Q, Zheng KI, Xu G*, et al.* PNPLA3 rs738409 is associated with renal glomerular and tubular injury in NAFLD patients with persistently normal ALT levels. *Liver international : official journal of the International Association for the Study of the Liver*. 2020; 40: 107-19.

[15] Alberti KG, Eckel RH, Grundy SM*, et al.* Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation*. 2009; 120: 1640-5.

[16] Liu WY, Zheng KI, Pan XY*, et al.* Effect of PNPLA3 polymorphism on diagnostic performance of various non-invasive markers for diagnosing and staging NAFLD. *Journal of Gastroenterology and Hepatology*. 2019; doi: 10.1111/jgh.14894.

[17] Angulo P, Hui JM, Marchesini G*, et al.* The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology (Baltimore, Md)*. 2007; 45: 846-54.

[18] Sterling RK, Lissen E, Clumeck N*, et al.* Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology (Baltimore, Md)*. 2006; 43: 1317-25.

[19] Kleiner DE, Brunt EM, Van Natta M*, et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology (Baltimore, Md)*. 2005; 41: 1313-21.

[20] EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *Journal of hepatology*. 2016; 64: 1388-402.

[21] Castera L, Forns X, Alberti A. Non-invasive evaluation of liver fibrosis using transient elastography. *Journal of hepatology*. 2008; 48: 835-47.

[22] Milovanovic Alempijevic T, Stojkovic Lalosevic M, Dumic I. Diagnostic Accuracy of Platelet Count and Platelet Indices in Noninvasive Assessment of Fibrosis in Nonalcoholic Fatty Liver Disease Patients. *Canadian journal of gastroenterology & hepatology*. 2017; 2017: 6070135.

[23] Kim D, Kim W. Association between body size-metabolic phenotype and nonalcoholic steatohepatitis and significant fibrosis. *Journal of gastroenterology*. 2019; doi: 10.1007/s00535-019-01628-z.

[24] Boursier J, Guillaume M, Leroy V*, et al.* New sequential combinations of non-invasive fibrosis tests provide an accurate diagnosis of advanced fibrosis in NAFLD. *Journal of hepatology*. 2019; 71: 389-96.

[25] Chan WK, Treeprasertsuk S, Goh GB*, et al.* Optimizing Use of Nonalcoholic Fatty Liver Disease Fibrosis Score, Fibrosis-4 Score, and Liver Stiffness Measurement to Identify Patients With Advanced Fibrosis. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*. 2019; doi: 10.1016/j.cgh.2019.03.006.

**TABLE TITLES**

**Table 1**. Baseline characteristics of patients with biopsy-proven NAFLD.

**Table 2**. Sensitivity, specificity, positive predictive value, negative predictive value, percentage of misclassification and indeterminate results, and percentage of correctly avoid liver biopsy, by using the nomogram alone and other different diagnostic approaches.

**Supplementary Table 1**. Baseline characteristics of patients.

**Supplementary Table 2**. Univariable and multivariable logistic regression analyses in the training set.

**Supplementary Table 3**. Subgroup analyses. Area under receiver operating characteristic curves (AUROC) with 95% confidence intervals of the nomogram and MACK-3, stratified either by presence of type 2 diabetes mellitus or by type of hypoglycemic treatment.

**FIGURE LEGENDS**

**Figure 1**. Nomogram to predict the presence of fibrotic NASH.To calculate the probabilityof having fibrotic NASH, trace a vertical line from each of the predictors’ axis to the first line (‘points’). Add the total points, and trace a vertical line from the ‘‘total points” axis to the risk axis to calculate the probability of having fibrotic NASH.

**Figure 2**. AUROCs of the non-invasive tests for the diagnosis of fibrotic NASH in both the training and validation sets.

**Figure 3**. Performances of two different sequential two-step approaches by using (A): the nomogram-LSM algorithm. A combination of the nomogram (first-line test) and liver stiffness measurement (LSM) (second-line test). (B): the MACK-3-LSM algorithm. A combination of MACK-3 (first-line test) and LSM (second-line test).