Title Page

Individualized polygenic risk score identifies NASH in the eastern Asia region: a derivation and validation study

Short Title: Polygene risk score for NASH.

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Abstract word count: 238 words

Total word count: 3,829 words (main text, *excluding* abstract, references and figure legends)

Number of figures/supplemental figures: 4/1

Number of tables/supplemental tables: 4/2

Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CAP, controlled attenuation parameter; CI, confidence interval; CK-18, cytokeratin-18 fragments; GGT, γ-glutamyl transpeptidase; GWAS, Genome-wide association studies; HOMA-IR, homeostasis model assessment of insulin resistance; HDL-C, high-density lipoprotein cholesterol; HSD17B13, 17-beta-hydroxysteroid dehydrogenase 13; LDL-C, low-density lipoprotein cholesterol; LSM, liver stiffness measurement; MBOAT7, membrane bound O-acyltransferase domain containing 7; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NAS-CRN, NASH-Clinical Research Network; NAS, NAFLD activity score; NFS, NAFLD fibrosis score; PNPLA3, patatin-like phospholipase domain-containing protein 3; SNP, single-nucleotide polymorphisms; TM6SF2, trans-membrane 6 superfamily member 2; TG, triglycerides; TC, total cholesterol.

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All authors contributed to the manuscript for important intellectual content and approved the submission.

Conflict of interest statement:

All authors: nothing to declare.

Financial support

This work was supported by grants from the National Natural Science Foundation of China (82070588), Research Grant of Tianqing for Liver Disease (TQGB20200097), High Level Creative Talents from Department of Public Health in Zhejiang Province (S2032102600032) and Project of New Century 551 Talent Nurturing in Wenzhou. GT is supported in part by grants from the School of Medicine, University of Verona, Verona, Italy. CDB is supported in part by the Southampton NIHR Biomedical Research Centre (IS-BRC-20004), UK. WK was supported by a National Research Foundation of Korea grant funded by the Korea government (2016R1D1A1B04934590), and the Korea Health Technology R&D Project through the Korea Health Industry Development Institute funded by the Ministry of Health & Welfare, Republic of Korea (HI17C0912).

Study Highlights

What is Known

The correct identification of patients at increased risk of NASH is a critical step in the assessment of NAFLD. There are few studies to date that have investigated whether a polygenic risk score predicts NASH.

What is New Here

This is the largest study that has ever developed a polygenic risk score for identifying NASH in patients with biopsy-proven NAFLD, and that we have validated the diagnostic performance of this risk score in an external validation cohort of NAFLD patients. Our results further confirm that the interaction of genetic and metabolic risk factors plays an important role in the development and progression of NAFLD.

Translational Impact

Our results may translate into clinical practice to guide the risk stratification of NAFLD and also stimulate further research into the pathogenic role of our risk score in NASH.

Keywords: NAFLD, NASH, single-nucleotide polymorphisms, *PNPLA3*, *HSD17B13*.

ABSTRACT

INTRODUCTION: Strong evidence indicates that multiple genetic and environmental risk factors play a role in the pathogenesis of non-alcoholic steatohepatitis (NASH). We aimed to develop and validate a novel nomogram, incorporating both genetic and clinical factors, for predicting NASH.

METHODS: A total of 1,070 Asian individuals with biopsy-confirmed non-alcoholic fatty liver disease (NAFLD) from two countries (China and South Korea) were recruited. The histological spectrum of NAFLD was classified according to the NASH clinical research network scoring system. The nomogram was developed in the Chinese training set (n=402); and then validated in both the Chinese internal validation set (n=136), and in the external Korean validation cohort (n=532), respectively.

RESULTS: Sex, metabolic syndrome, insulin resistance, serum aspartate aminotransferase levels, and *PNPLA3* (rs738409) and *HSD17B13* (rs72613567) genetic variants were strongly associated with NASH. Based on their regression coefficients, we developed a nomogram with a good discriminatory ability (area under the ROC curve [AUROC]: 0.81, 95% CI 0.77-0.85), as well as good calibration (Hosmer-Lemeshow test, p=0.794) for identifying NASH. In the two validation cohorts, the nomogram showed high AUROCs (internal validation set: 0.80, 95% CI, 0.72-0.88; external validation cohort: 0.76, 95% CI, 0.72-0.80), as well as good calibration.

DISCUSSION: Our newly developed and externally validated nomogram,

incorporating both genetic and clinical risk factors, may be conveniently used to predict NASH. Further validation studies in other ethnic groups are warranted to confirm its diagnostic utility to identify NASH, among biopsy-proven NAFLD patients.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become the commonest cause of chronic liver disease in many parts of the world, affecting up to a quarter of the general adult population.[1] The histopathological spectrum of NAFLD ranges from simple steatosis (NAFL) to non-alcoholic steatohepatitis (NASH), advanced fibrosis and cirrhosis.[2] NASH is also becoming one of the main indications for liver transplantation (LT) amongst the registrants on LT waiting lists both in the United States and in Europe. [3,4] Approximately 20% of patients with NASH can progress to cirrhosis and hepatocellular carcinoma requiring LT.[5] Additionally, NASH is significantly associated with an increased risk of developing important extra-hepatic complications, such as cardiovascular disease (which represents the leading cause of death in this patient population) and chronic kidney disease.[6,7]

The correct identification of patients at increased risk of NASH is a critical step in the assessment of NAFLD.[8] Treatment of NASH is a major focus of drug development worldwide.[9,10] Although, currently, there are no Food and Drug Administration - approved therapies for NASH, there are ~196 drugs being evaluated for the treatment of NASH and many phase 2 and phase 3 randomized controlled trials, are ongoing.[11] To date, liver biopsy and histological examination of liver tissue remains the reference method for diagnosing NASH. However, liver biopsy is an invasive method that cannot be used for screening the general population. Therefore, a major challenge is how to accurately and non-invasively identify patients with NASH, who may

potentially benefit from early lifestyle intervention and future pharmacological treatment.

Metabolic disorders, such as obesity, type 2 diabetes mellitus (T2DM) and metabolic syndrome (MetS), are important clinical risk factors for NASH,[12] but not all individuals with these risk factors have NASH. Familial clustering of NAFLD suggests that this disease is also strongly influenced by heritable genetic factors.[13] Genome-wide association studies (GWAS) have also showed that some genetic variants play an important role in the development and progression of NAFLD.[14-16] Patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) genetic variant is the strongest genetic risk factor for the development of NASH.[17] Indeed, studies have shown that individuals carrying the *PNPLA3* (rs738409) variant have a ~threefold increased likelihood of having NASH.[18-20] Moreover, single-nucleotide polymorphisms (SNPs) in the trans-membrane 6 superfamily member 2 (*TM6SF2* rs58542926), membrane bound O-acyltransferase domain containing 7 (*MBOAT7* rs641738), and 17-beta-hydroxysteroid dehydrogenase 13 (*HSD17B13* rs72613567) genetic variants are also associated with greater susceptibility to NASH.[21-23]

Strong evidence indicates that the interaction between the genetic background and metabolic risk factors plays an important role in the pathogenesis of, and disease progression in NAFLD.[24] For example, the *PNPLA3* (rs738409) variant has a stronger effect on liver injury in obese individuals than in lean individuals.[25] Moreover,

polygenic risk scores adjusted for conventional clinical risk factors may have the potential to guide and inform the care of patients with NAFLD.[24] On this background of evidence, the two major aims of our study were as follows: 1) to identify relevant genetic and clinical risk factors associated with NASH; and 2) to develop and validate a novel nomogram for predicting NASH in a large multi-national cohort of Asian patients with biopsy-proven NAFLD.

METHODS

Study population and design

We conducted a cross-sectional study involving two cohorts of adult patients with biopsy-proven NAFLD from China and South Korea. The primary cohort comprised 1,022 potentially eligible Chinese patients diagnosed with suspected NAFLD (based on the presence of hepatic steatosis on imaging methods and/or elevated serum liver enzymes) between December 2016 and October 2019 at the First Affiliated Hospital of Wenzhou Medical University in Wenzhou (China). Exclusion criteria were: (i) significant alcohol consumption (≥140 g/week in men or ≥70 g/week in women); (ii) presence of viral hepatitis, autoimmune hepatitis, drug-induced liver injury, or other known chronic liver diseases; (iii) incomplete clinical or genetic data; and (iv) hepatic steatosis <5% on liver histology. Between January 2013 and May 2020, an independent validation cohort of 852 potentially eligible patients with NAFLD from Seoul National University Seoul Metropolitan Government Boramae Medical Center in Seoul (South Korea) was also recruited. The inclusion and exclusion criteria were

consistent with those of the primary Chinese cohort. As a result of the aforementioned exclusion criteria, a total of 1,070 NAFLD patients with complete data were included in the study. More detailed information about the two patient cohorts is shown in **Supplementary Table 1**.

The study protocol was approved by the local ethics committees of the two hospitals. All procedures involving the participants were performed in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration. Written informed consent was obtained from each subject after full explanation of the purpose and nature of all procedures.

Clinical and biochemical data

Clinical and biochemical data were obtained from all participants within 48 hours from liver biopsy. Blood samples were taken in fasting conditions. Body mass index (BMI) was calculated using the formula weight (kilograms) divided by height (meters) squared. Central obesity was defined as waist circumference ≥ 90 cm in men and ≥ 80 cm in women in the Asian population.[26] Insulin resistance was estimated using the homoeostasis model assessment of insulin resistance (HOMA-IR) and defined as HOMA-IR > 2.5.[27] T2DM was diagnosed as either self-reported history of disease, a fasting glucose level ≥ 7.0 mmol/L, hemoglobin A1c ≥ 6.5% (≥ 48 mmol/mol) or use of any anti-hyperglycemic drugs. Hypertension and dyslipidemia were diagnosed according to consensus criteria.[28] MetS was defined as having at least

three of the following metabolic risk factors: central obesity, increased blood pressure (systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg or use of any antihypertensive drugs), increased fasting glucose (≥5.6 mmol/L or use of any antihyperglycemic agents), high triglycerides (>1.7 mmol/L or use of any lipid-lowering drugs) and low high-density lipoprotein cholesterol levels (<1.03 mmol/L in men and <1.29 mmol/L in women, or use of any lipid-lowering drugs).[26,29] Methodological details for measurement of plasma cytokeratin-18 fragments (CK-18 neoepitope M30) levels have been reported previously.[30] FIB-4 and NAFLD fibrosis score (NFS) were calculated using published formulas.[8] Controlled attenuation parameter (CAP) and liver stiffness measurement (LSM) were measured by two experienced operators using vibration-controlled transient elastography (Fibroscan®; Echosens, Paris, France), according to the manufacturer's recommendations.

Genetic analysis

Genotyping assays for *PNPLA3* (rs738409)*, HSD17B13* (rs72613567)*, TM6SF2* (rs58542926), and *MBOAT7* (rs641738) variants on human peripheral blood leukocytes were carried out using the MassARRAY, Sanger sequencing, or TaqMan assays platform according to the manufacturer's protocol.[31,32]

Liver histology

Percutaneous liver biopsy was performed under ultrasound guidance. Liver histology assessment was undertaken by experienced liver histopathologists (who were blinded

to the clinical and genetic data of participants) according to the NASH-Clinical Research Network (CRN) Scoring System.[33] The NAFLD activity score (NAS) was calculated as the sum of three histological components, including liver steatosis (0-3), ballooning (0-2), and lobular inflammation (0-3). Liver fibrosis was staged as zero to 4 according to the Brunt's histologic criteria.[34] NAFLD was defined as the presence of hepatic steatosis in more than 5% of hepatocytes. NASH was diagnosed based on an overall pattern of histological hepatic injury consisting of macrovesicular steatosis, inflammation, and hepatocellular ballooning.[33,35]

Statistical analysis

Continuous variables were expressed as means ± SD or medians with interquartile ranges (IQRs), and compared using either the unpaired Student's *t*-test or the Mann-Whitney U test as appropriate. Categorical variables were expressed as number (percentages) and compared using the chi-squared test or the Fisher's exact test as appropriate.

For the development of our nomogram, the primary Chinese cohort was randomly assigned in a 3:1 ratio to training and internal validation sets, using a split-sample method by an experienced statistician. Multivariable logistic regression analysis began with the variables selected from univariable analysis ($P < 0.10$). Stepwise selection was applied by using the likelihood ratio test with Akaike's information criterion as the stopping rule. To provide the clinician with a quantitative tool to

determine the individual probability of NASH, we built the nomogram on the basis of multivariable logistic analysis results obtained in the training set. The accuracy of this novel diagnostic model was subsequently evaluated both in the internal validation set and an independent external validation cohort. The diagnostic cut-offs for the nomogram, corresponding to the 90% sensitivity and 90% specificity thresholds for NASH, were calculated in the training set. The sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), and the grey zone of the model were calculated at each cut-off. The discrimination of the model was evaluated by calculating the area under the receiver operating characteristic curve (AUROC). The model calibration was assessed by the calibration curve and the Hosmer-Lemeshow goodness of fit test. Statistical analyses were two-sided and significance was set at p <0.05. All statistical tests were performed using R (Version 3.3.1 The R Foundation).

Results

Baseline characteristics of patients

A total of 1,070 patients with biopsy-confirmed NAFLD from two tertiary hepatology centers were included in the study (**Fig. 1**). In the primary Chinese cohort (n=538), patients were randomly assembled into a “training set” (n=402) and a "validation set" (n=136). At the time of liver biopsy, patients had a median age of 42 years in the training set, and a median age of 43 in the validation set. The prevalence of NASH was 42.5% in the training set and 36.8% in the validation set, respectively. In the

external validation cohort (involving 532 South Korean patients), the median age was 54 years and the prevalence of NASH was 33.5%. The baseline characteristics of the primary and validation cohorts are summarized in **Table 1**. The characteristics of the patients according to NASH status in the training set are shown in **Supplementary Table 2.**

The frequency distributions of *PNPLA3* (rs738409)*, HSD17B13* (rs72613567*),* *TM6SF2* (rs58542926), and *MBOAT7* (rs641738) genotypes were all in Hardy-Weinberg equilibrium (training set: p=0.201, 0.664, 0.781 and 0.755, respectively; external validation cohort: p=0.464, 0.128, 0.956 and 0.999).

Development of an individualized risk score

Through univariable analyses, age, sex, BMI, MetS, serum liver enzymes (ALT, AST, γ-GT), albumin, HOMA-estimated insulin resistance, as well as the *PNPLA3* rs738409 and *HSD17B13* rs72613567 genetic variants were selected for developing an individualized risk score to identify NASH (all p<0.1) (**Supplementary Table 2**). In multivariable regression analyses, there was a strong association between NASH and sex, presence of MetS, HOMA-IR >2.5, increased AST levels (≥40 U/L), and the *PNPLA3* rs738409 and *HSD17B13* rs72613567 genetic variants. Finally, an individualized risk score for the non-invasive identification of NASH was developed based on the regression coefficients (**Fig. 2**). The formula for the risk score was as follows: 0.548 × sex (female =1; male =0) + 0.467 × MetS (yes =1; no =0) + 1.909 ×

elevated AST levels (AST ≥40 =1; AST <40 U/L =0) + 1.074 × insulin resistance (HOMA-IR >2.5 =1; HOMA-IR ≤2.5 =0) + 0.581 × *PNPLA3* (rs738409) genotype (GC =1; CC or GG =0) + 1.228 × *PNPLA3* (rs738409) genotype (GG =1; CC or GC =0) + 0.607 × *HSD17B13* (rs72613567) genotype (AA or -/A =1; -/- =0). For example, for a woman whose serum AST level was 100 U/L, HOMA-IR level was 3.0, and having MetS, *PNPLA3* (rs738409) GG genotype, and *HSD17B13* (72613567) AA genotype, her total points score was 6 and her probability of having NASH was 94%.

Diagnostic performance of the nomogram in the primary Chinese cohort

The AUROCs for the nomogram were 0.81 (95% CI 0.77-0.85) for the training set **(Fig. 3A)** and 0.80 (95% CI 0.72-0.88) for the validation set **(Fig. 3B)**. The calibration curve of the nomogram for the probability of NASH showed good agreement between prediction and observation in both the training and validation sets **(Fig. 3D, E)**. The Hosmer-Lemeshow test showed a non-significant statistic (training set: p= 0.794; validation set: p=0.519), indicating that there was no departure from perfect fit. With the specific aim of identifying the most accurate nomogram cut-off values for diagnosing NASH, we used dual cut-off values of <2.20 (sensitivity=0.90 in the training set) and >4.10 (specificity=0.91 in the training set), respectively. In the training set, the cut-off value <2.20 had a negative predictive value (NPV) of 0.87 to rule out NASH, whereas the cut-off value >4.10 had a positive predictive value (PPV) of 0.76 to rule in NASH. Similarly, in the internal validation set, the cut-off value

<2.20 had an NPV of 0.88 to rule out NASH, whereas the cut-off value >4.10 had a PPV of 0.77 to rule in NASH **(Table 2)**. In addition, we found that the nomogram scores increased significantly across the histologic grades of steatosis, ballooning, lobular inflammation, and fibrosis (**Fig. 4)**.

FIB-4 and NFS are widely used as non-invasive diagnostic tests for diagnosing liver fibrosis in NAFLD patients, and plasma CK-18 levels are one of the most widely studied modalities for diagnosing NASH. We compared the performance of our nomogram with FIB-4, NFS, and plasma CK-18 for diagnosing NASH. As shown in **Table 4**, the discriminatory ability of our nomogram was superior to plasma CK-18, NFS and FIB-4 scores. LSM and CAP values were measured using FibroScan in a subset of 357 NAFLD patients. We have also compared the performance of our nomogram with that of LSM and CAP values. The AUROC of the nomogram was higher than those of LSM and CAP (**Table 4**).

External validation of the nomogram

The nomogram yielded an AUROC of 0.76 (95%CI, 0.72-0.80) in the external validation cohort from South Korea (**Fig. 3C**). Good calibration was observed for the probability of NASH (**Fig. 3F**), and the Hosmer-Lemeshow test showed a non-significant statistic (p=0.999). Using the aforementioned dual cut-off approach, the NPV in the validation cohort was of 0.91, and 38.7% of the patients were in the 'grey' zone between the two cut-off points **(Table 2)**. Similar to our results in the primary

Chinese cohort, the nomogram scores increased progressively across the histologic grades of hepatic steatosis, ballooning, lobular inflammation, and fibrosis in the external validation cohort (**Supplementary Fig. 1**).

Subgroup analysis

We also tested the performance of our novel nomogram by subgroup analyses (stratified by sex and age thresholds) both in the primary Chinese cohort and in the South Korean validation cohort. As shown in **Table 3**, the nomogram performed well in patients with and without pre-existing T2DM as well as in those with and without MetS. Among men with NAFLD, the AUROCs of the nomogram were 0.81 (0.76-0.85) in the Chinese cohort and 0.76 (0.70-0.81) in the Korean cohort, respectively. Among women with NAFLD, the AUROCs of the nomogram were 0.77 (0.69-0.84) in the Chinese cohort and 0.74 (0.68-0.79) in the Korean cohort. Stratifying by age groups, the AUROCs of the nomogram for patients younger than 40 years were 0.80 (0.74-0.86) in the Chinese cohort and 0.79 (0.71-0.86) in the Korean cohort; the AUROCs for patients aged between 40 years and 60 years were 0.81 (0.75-0.86) in the Chinese cohort and 0.78 (0.71-0.83) in the Korean cohort; and the AUROCs for patients older than 60 years were 0.67 (0.48-0.86) in the Chinese cohort and 0.72 (0.65-0.78) in the Korean cohort, respectively. The diagnostic performance of our nomogram was slightly diminished in women and in older (>60 years) patients in both the Chinese and the Korean cohorts.

Discussion

In this multicenter study involving a cohort of 1,070 middle-aged individuals with biopsy-confirmed NAFLD from both China and South Korea, we developed and validated a clinical and genetic risk factors-based nomogram for identifying NASH. Our novel nomogram (including sex, MetS, HOMA-IR, serum AST level, *PNPLA3* and *HSD17B13* genotypes in its equation) had a good discriminatory capacity and calibration for identifying NASH in both the training and validation cohorts. This nomogram performed well in both patients with and without pre-existing T2DM, as well as in those with and without MetS. The accuracy of the nomogram was (slightly) diminished in older participants. Our nomogram was positively associated with all individual histologic scores of NASH, including the fibrosis stage. These results further confirm that the interaction of genetic and metabolic risk factors plays an important role in the development and progression of NAFLD.

As NAFLD affects up to a quarter of the general population worldwide,[1] millions of patients worldwide, who are at risk of NAFLD progression, would benefit from treatment that is focused on effecting regression of NASH. Recently, a nationwide matched cohort study in Sweden has shown that all histological stages of NAFLD were associated with significantly increased overall mortality, and this risk increased progressively with worsening NAFLD histology[36] (compared with matched controls, significant excess mortality risk was observed with simple steatosis (8.3/1,000 person-year [PY]) and NASH (13.4/1,000 PY); compared with those with simple steatosis,

the multivariable-adjusted hazard ratio for overall mortality was increased in patients with NASH [HR 1.14; 95% CI 1.03-1.26]).[36] Moreover, compared with matched controls, the mortality rate from cardiovascular causes was increased in those with NASH (absolute rate difference 2.7/1,000 PY), and compared with those with simple steatosis, the 20-year absolute excess risk of cardiovascular mortality was higher in patients with NASH (4.4%, $p<0.05$).[36]

In recent years, a number of SNPs have been reported to be associated with susceptibility to NASH.[24] The rs738409 C>G variant in the *PNPLA3* gene is the first and strongest genetic variant found to be associated with the susceptibility to NASH.[17] *PNPLA3* involves in lipid droplet remodeling in hepatocytes and retinol production by hepatic stellate cells.[37] A recent GWAS confirmed that the rs738409 variant in the *PNPLA3* gene was a risk factor across the entire histological spectrum of NAFLD.[14] Our study has also confirmed that the *PNPLA3* rs738409 was the most robustly associated genetic variant associated with NASH among the four SNPs that were tested in this study. *HSD17B13* is a lipid droplet-associated protein, expressed predominantly in the liver, implying a liver-specific function.[38] It has been reported that inactivating variants in the *HSD17B13* gene are associated with a reduced risk of chronic liver disease among whites' individuals.[23,39] However, we observed an inverse allelic association. As all our study participants are from East Asia, ethnic differences between patients might partly explain the results. A differential allele effect direction of genetic variants discovered by GWAS in subjects of different ethnicities is not

uncommon.[40] Lee et al. recently reported that the associations of apolipoprotein(a) (*LPA*) SNPs with size of apolipoprotein(a) isoforms, lipoprotein(a) and oxidized phospholipids on apolipoprotein B-100 levels are variable and ethnicity-specific.[40] In addition, *HSD17B13* deficiency in mice models did not reproduce the protective effect of *HSD17B13* loss-of-function mutants seen in human NAFLD.[41] Interestingly, *HSD17B13* deficiency induced weight gain in mice fed regular chow, which is contrary to previous findings.[41]

Emerging data indicate that polygenic risk scores (PRS) have the potential to guide and inform the care of patients with NAFLD. Costanzo et al. reported a risk score based on *TM6SF2*, *GCKR*, *PNPLA3* and *MBOAT7* genes could accurately identify patients with ultrasound-detected NAFLD from the general population.[42] Krawczyk et al. found an increasing risk of hepatic steatosis and fibrosis with increasing number of *PNPLA3*, *TM6SF2* and *MBOAT7* risk alleles in NAFLD patients.[43] Moreover, León-Mimila et al. studied 130 Mexican Mestizo subjects with severe obesity undergoing bariatric surgery, and found a PRS that included the *PNPLA3*, *LYPLAL1*, *PPP1R3B* and *GCKR* genes, was associated with hepatic steatosis; although this score did not predict NASH (AUROC=0.56, P=0.219).[44] There are few studies to date that have investigated whether a PRS predicts NASH. In contrast to previous studies, we recruited Asian patients with biopsy-proven NAFLD and found that the combination of polygenic and clinical risk factors could accurately identify NASH.

Previous studies have found that the *PNPLA3* variant exerted its adverse hepatic effects predominantly in obese patients compared to lean individuals.[24,25] It has also been observed that the presence of insulin resistance, T2DM or MetS affects the interaction between genetic and environmental risk factors.[24] Barata et al. recently found that the *PNPLA3* rs738409 variant significantly interacts with insulin resistance, BMI, and plasma glucose and triglycerides levels to worsen hepatic steatosis in non-diabetic individuals carrying the G allele.[45] The mechanism by which these modifiable metabolic traits interact with genetic variants to influence the risk of NASH remains to be clarified.

Through our multivariable logistic regression analyses, we demonstrated that female sex, MetS, HOMA-estimated insulin resistance, elevated serum AST levels, and presence of *PNPLA3* (rs738409) and *HSD17B13* (rs72613567) genetic variants were strongly associated with NASH. Overall, therefore, we believe that our results may contribute to better understanding of the polygenic regulation of NASH and the complex interaction between genetic and environmental risk factors. The major strengths of our study are that this is the largest study that has ever developed a polygenic risk score for identifying NASH in patients with biopsy-proven NAFLD, and that we have validated the diagnostic performance of this risk score in an external validation cohort of NAFLD patients.

The major limitation of our study is that participants were all from the East Asia and,

therefore, our results may not be applicable to other ethnic groups who have other metabolic risk factors in particular. Further studies are needed to test the diagnostic accuracy of our novel nomogram in non-Asian individuals with NAFLD. Genetic testing is not widely available and not easy to perform, which makes widespread implementation difficult in clinical practice. However, genetic testing has been demonstrated to have a role in genetic counseling, prevention strategies and treatments, in other fields of medicine, including oncology, cardiology and psychiatry.[46,47] Genetic testing may also in the future have a role in genetic counseling in NAFLD. In addition, there were some differences in the demographic characteristics between the Chinese and Korean cohorts, reflecting the heterogeneity of the populations with NAFLD. We have tested the performance of our nomogram in subgroup analyses and found that the diagnostic performance of our nomogram was slightly diminished in women and in older (>60 years) participants in both cohorts of patients. Finally, there is a grey zone extending from 38% to 49% when assessing the diagnostic performance of our nomogram for predicting NASH. However, almost all non-invasive tests of NAFLD are currently limited by a clear grey zone due to the use of two-cutoff thresholds.[48] Although there is a grey zone in our nomogram, liver biopsies could be correctly avoided in our study in approximately 50% of patients by using the score. In addition, a 2-step approach was recently reported to reduce indeterminate or discordant results while maintaining accuracy (the second test is used if a result in the grey zone is obtained from first test, and liver biopsy is performed if the result is in the grey zone for the second test).[49] By using this 2-step

approach, the need for liver biopsy would be reduced significantly without much effect on the percentage of misclassifications.[50] Further studies are needed to evaluate whether the combination of our nomogram with other non-invasive scores facilitates the stratification of disease severity in NAFLD.

In conclusion, we have developed and validated a novel nomogram incorporating both genetic and clinical risk factors that accurately identifies NASH in a large cohort of Asian patients with biopsy-proven NAFLD. These results may translate into clinical practice to guide the risk stratification of NAFLD and also stimulate further research into the pathogenic role of our risk score in NASH.

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FIGURE LEGENDS

Figure 1. The flowchart for the study.

Figure 2. Nomogram to identify the presence of NASH. To calculate the probability of having NASH, trace a vertical line from each of the predictors' axis to the first line. Add the total points and trace a vertical line from the "total points" axis to the risk axis to calculate the probability of having NASH.

Figure 3. Diagnostic performance of the nomogram for the diagnostic of NASH. (A) AUROC of the training set; (B) AUROC of the internal validation set; (C) AUROC of the external validation cohort; (D) calibration curve of the training set; (E) calibration curve of the internal validation set; and (F) calibration curve of the external validation cohort.

Figure 4. Boxplot of the score versus histopathological severity of the primary Chinese cohort: (A) steatosis grade, (B) lobular inflammation grade, (C) ballooning grade, and (D) fibrosis stage.

Supplementary Figure 1. Boxplot of the score versus histopathological severity of the external (Korean) validation cohort: (A) steatosis grade, (B) lobular inflammation grade, (C) ballooning grade, and (D) fibrosis stage.