Title Page

Title: Effect of *PNPLA3* polymorphism on diagnostic performance of various non-invasive markers for diagnosing and staging NAFLD

Short Title: *PNPLA3* genotype and non-invasive diagnostic markers

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Abbreviations

NAFLD = nonalcoholic fatty liver disease, NASH = nonalcoholic steatohepatitis, ALT = alanine aminotransferase, AST = aspartate aminotransferase, *PNPLA3*= Patatin-like phospholipase domain-containing protein 3, CK18 = cytokeratine-18, FIB-4 = Fibrosis-4, GGT = gamma-glutamyl transferase, BMI = body mass index, FLI = fatty liver index, HSI = hepatic steatosis index, AUROC = area under the receiver operator characteristic curve, CI = confidence interval

Contributors

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Abstract

Background and Aim: Patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) I148M (rs738409) genotype influences clinical/biochemical characteristics in patients with nonalcoholic fatty liver disease (NAFLD), but whether *PNPLA3*-I148M (rs738409) genotype also influences the diagnostic performance of non-invasive diagnostic tests for NAFLD is uncertain. Our aim was to investigate the differences in diagnostic performance of non-invasive diagnostic tests for NAFLD according to *PNPLA3*-I148M (rs738409) genotype.

Methods: 58 healthy controls and 349 patients with biopsy-proven NAFLD were included. Areas under operating characteristics (AUROCs) were calculated for predicting hepatic steatosis [fatty liver index (FLI), hepatic steatosis index (HSI)]; NASH [cytokeratin-18 (CK18) M30 and M65] and significant fibrosis ($\geq$F2 fibrosis) [Fibrosis-4 (FIB-4) and BARD], stratifying by rs738409 genotypes (CC and CG+GG groups).

Results: FLI and HSI showed good diagnostic performance for diagnosing steatosis only in the CG+GG group with AUROCs ranging from 0.819 to 0.832 respectively. CK18 M30 (AUROC=0.688) and M65 (AUROC=0.678) had suboptimal performance for diagnosing NASH in the CG+GG group, whereas both had good performance (AUROC=0.814 and 0.813, respectively) in the CC group. BARD score showed good performance in the CG+GG group compared to the CC group (AUROC=0.805 and 0.532, respectively). FIB-4 had suboptimal performance in the CG+GG group, and good performance in the CC group (AUROC =0.662 and 0.801, respectively).

Conclusions: Diagnostic performance of non-invasive tests for NAFLD varied markedly according to *PNPLA3* genotypes. Clinicians should be aware that *PNPLA3* genotype limits the clinical utility of non-invasive diagnostic tests for diagnosing NAFLD.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has become a major health burden, affecting ~25-30%
of the general adult population worldwide.[1] However, the prevalence of NAFLD varies widely
across different geographic regions, with highest prevalence in the Middle East and South
America and lowest in Africa.[1] NAFLD represents a spectrum of liver diseases from simple
steatosis (NAFL) to steatohepatitis (NASH) with varying degrees of fibrosis, and cirrhosis.[2]
NAFLD is much higher in people with type 2 diabetes and is also expected to become the most
important cause of hepatocellular carcinoma and liver transplantation worldwide.[3-5] Liver biopsy,
which is regarded as the 'gold standard' for the diagnosis of NAFLD, has some well-known
limitations, such as low patient acceptance, acute complications, sampling errors and high
cost(s). This has generated a growing interest for non-invasive markers for assessing the
presence and severity of NAFLD (i.e. simple steatosis to NASH and significant fibrosis).

Large genome-wide association studies have provided useful insights into the pathogenesis of
NAFLD showing that the common genetic variants of *PNPLA3* (encoding for patatin-like
phospholipase domain-containing 3 protein) are strongly associated with an increased risk of
NASH and cirrhosis.[6-8] Currently, a number of studies have identified a subgroup of *PNPLA3*-
148M carriers with NAFLD who do not have insulin resistance and other typical features of
metabolic syndrome, suggesting that NAFLD pathophysiology may be different in these
subjects.[7, 9, 10] Consequently, these data highlight the need to determine whether non-invasive
diagnostic tests perform equally well for diagnosing and staging NAFLD in patients with
different *PNPLA3* genotypes. Many studies have incorporated *PNPLA3* genetic variants in their
non-invasive scoring systems to aid the risk prediction of NAFLD.[11, 12] However, single-

nucleotide polymorphisms (SNPs) have been used only as a risk component in these scoring systems and whether there are differences in the diagnostic performance of non-invasive tests for diagnosing and staging NAFLD in different *PNPLA3* genotypic groups is currently uncertain.

The aim of this study was to investigate possible differences in diagnostic performance of non-invasive diagnostic tests for hepatic steatosis, NASH and fibrosis according to *PNPLA3* rs738409 genotypes. In particular, we have tested the diagnostic performance of fatty liver index (FLI) and hepatic steatosis index (HSI) for predicting steatosis, cytokeratin-18 (CK18) M30 and M65 levels for predicting NASH, and Fibrosis-4 (FIB-4) and BARD for predicting significant liver fibrosis (i.e. ≥F2 fibrosis on histology).

METHODS

Study population

In this study, participants were recruited from the well-characterized Prospective Epidemic Research Specifically Of NASH (PERSONS) cohort from 2017 to 2019. Inclusion criteria are as follows: (1) between 18-75 years of age; (2) provided informed written consent. Exclusion criteria are as follows: (1) excessive alcohol ingestion (>20 g/day over the past 2 years); (2) presence of chronic liver diseases (other than NAFLD) such as viral hepatitis (based on measurement of serum markers for viral B or C hepatitis) and autoimmune hepatitis (based on serum autoantibodies and histology); (3) chronic use of drugs that may result in significant liver damage (i.e. more than 6 months and serum aminotransferase levels more than three times of upper limit of normal); (4) pregnant or lactating women; and (5) primary liver cancers or malignancies of other origins. As a consequence of the aforementioned criteria, 407 participants

were included in the study (349 with NAFLD and 58 non-steatotic healthy controls, all of which confirmed by liver histology). The protocol for the purpose of this study was approved by the internal review board for ethics at the First Affiliated Hospital of Wenzhou Medical University (2016-246, 1 December 2016) and the same protocol was registered in the Chinese Clinical Trial Registry (ChiCTR-EOC-17013562).

Liver histology assessment

Specimens from liver biopsy were scored by a single liver pathologist (X.D. Wang), who was blinded to participants' clinical data. Histological parameters were defined based on the NAFLD Activity Score (NAS) system by Kleiner et al.[13] NAFLD was considered by observation of steatosis grade >5% without secondary cause of fatty liver, and NASH was diagnosed as presence of NAS≥5 according to the histological scoring system for NAFLD developed by the NASH Clinical Research Network.[13] Significant fibrosis was defined by fibrosis ≥F2 on histology.

Analysis of the PNPLA3 rs738409 polymorphism

Genotyping of rs738409 was evaluated by the MassARRAY platform (Agena Bioscience, San Diego, CA, USA). For the purpose of genotyping, each sample used approximately 20 ng of genomic DNA extracted from peripheral blood leukocytes. Locus-specific PCR and detection primers were designed using Assay Design Suite v3.1. MALDI-TOF mass spectrometry was used to detect allele type following DNA amplification by multiplex PCR.

Measurement of serum cytokeratin-18

Circulating levels of CK-18 M30 and M65 were measured by commercial ELISA kits provided by Herui Biomed Company Limited, Suzhou, China. Both intra-assay and inter-assay coefficients of variation were <15%.

Indices of non-invasive markers

Briefly, HSI[14], FLI[15], FIB-4 index[16], and BARD score[17] were calculated using published formulas.

Statistical analysis

Statistical analysis was performed using R software (version 3.5.2, R Foundation for Statistical Computing, Vienna, Austria) and MedCalc (version 11.4.2.0, MedCalc software). Continuous variables were expressed as mean ± SD and compared using the Student's *t*-test. Categorical variables were expressed as frequency (%) and compared using the chi-square test. Receiver operating characteristic (ROC) curves, plotting sensitivity against 1–specificity, were also used to assess the diagnostic performances of non-invasive tests. Areas under ROC curve (AUROC) with 95% confidence intervals (95% CI) were calculated under non-parametric (distribution free) assumption. Optimal cut-off values were calculated to maximise sensitivity and specificity. For each cut-off, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were reported. P values <0.05 were considered to be statistically significant.

RESULTS

Baseline characteristics

A total of 407 participants met the enrolment criteria and consisted of 58 biopsy-confirmed healthy controls and 349 patients with biopsy-confirmed NAFLD. Baseline clinical and biochemical characteristics of participants are listed in Table 1. Patients with NAFLD had higher FLI (43.8 *vs*. 62.8, P<0.001) and HSI (35.4 *vs*. 40.4, P<0.001) compared to healthy controls. In addition, the two groups significantly differed in terms of age, BMI, waist circumference, serum ALT, AST and uric acid levels.

Patients with NAFLD were stratified into non-NASH (n=186) and NASH (n=163) subgroups according to the NAS score. Table 2 shows the baseline characteristics of these two groups of patients. Serum levels of CK18 M30 (199.4 U/L *vs*. 461.3 U/L, *P* <0.001) and CK18 M65 (346.8 U/L *vs*. 762.5 U/L, *P* <0.001) were significantly higher in the NASH subgroup. In addition, age, sex, BMI, serum ALT, AST, uric acid, total cholesterol and LDL cholesterol levels were also statistically different between the two patient groups.

Patients with NASH were stratified by significant fibrosis status (Table 3). 126 of 163 NASH patients had non-significant fibrosis. Mean BARD score (0.9 ± 1.0 *vs*. 1.9 ± 1.2, *P* <0.001) and FIB-4 (0.9 ± 0.4 *vs*. 1.2 ± 0.9, *P* =0.004) were significantly higher for the significant fibrosis subgroup. Moreover, the proportion of subjects with diabetes, as well as the levels of HbA1c, red blood cell count and hemoglobin were also significantly different between two groups of patients.

Results of SNP genotyping show that the proportion of rs738409 CC group was 42.9% in healthy controls, and 28.4% in NAFLD subjects (Table 1). In the non-NASH and NASH subgroups, the proportion of rs738409 CC group was 32% and 24.2%, respectively (Table 2). Among NASH

patients, for the non-significant fibrosis subgroup and the significant fibrosis subgroup, the proportions with CC genotype were 25.6% and 18.8%, respectively (Table 3).

Diagnostic performance of non-invasive markers

To investigate the diagnostic performance of non-invasive markers in different *PNPLA3* genotypes, subjects were divided into two groups: the rs738409 CC group and the rs738409 CG+GG group (Figure 1).

After pooling healthy controls and NAFLD subjects, the diagnostic performance of FLI and HSI were investigated for hepatic steatosis. The results showed that these two non-invasive markers had good diagnostic performance for diagnosing steatosis only in the rs738409 CG+GG group (Table 4). For example, the AUROCs for HSI and FLI were 0.819 and 0.832 respectively (*P* <0.001 for both) for subjects carrying the G allele of rs738409 variant.

Inconsistent diagnostic performances were observed for differentiating NASH from non-NASH. Table 4 shows that CK18 M30 (AUROC =0.688, *P* <0.001) and M65 (AUROC =0.678, *P* <0.001) had suboptimal performances for diagnosing NASH in the rs738409 (CG+GG) group, whereas CK18 M30 and M65 had good performance (AUROC =0.814 *vs.* 0.813, *P* <0.001) in the rs738409 CC group.

Table 4 shows the diagnostic performances of non-invasive fibrosis markers by different *PNPLA3* genotypic groups. The BARD score had better performance in the CG+GG genotype group compared to the CC genotype group (AUROC =0.805 *vs.* 0.532; *P* <0.001 and 0.809,

respectively). FIB-4 test performed better in the CC group (AUROC =0.662 *vs.* 0.801; *P* =0.009 and 0.018, respectively). while it had suboptimal performance patients in the CG+GG group,

Diagnostic cut-offs of non-invasive markers

As mentioned above, discrepancies in AUROCs were observed between non-invasive markers for diagnosing liver steatosis, NASH and significant fibrosis with NASH, when subjects were grouped by *PNPLA3* genotypes. In the same way, we examined the optimal diagnostic cut-offs for each non-invasive marker.

As shown in Table 4, the cut-off of FLI for diagnosing liver steatosis was 42 for the rs738409 CC group and 50 for the rs738409 CG+GG group. The specificity of these cut-offs was 52.17% in the rs738409 CC group and 80.65% in the rs738409 CG+GG group. In contrast, HSI had almost the same cut-off for diagnosing NAFLD between the two different *PNPLA3* genotypes (38 in CC and 39 in CG+GG groups).

In non-NASH and NASH groups, the optimal CK18 M30 cut-offs were 172 U/L and 196 U/L for the two patient groups, respectively, whereas the optimal cut-offs for CK18 M65 were 217 U/L and 207 U/L, respectively. The cut-offs for these aforementioned biomarkers were relatively consistent both in the rs738409 CC and in the CG+GG groups.

Consistent optimal cut-offs for BARD score were observed in diagnosing significant fibrosis among patients with NASH (Table 4). However, the optimal FIB-4 cut-offs showed

inconsistency between the CG+GG group and the CC group (0.872 and 0.535, respectively; P =0.009 and 0.018, respectively).

DISCUSSION

The novel findings of our cross-sectional study are that the diagnostic performance of non-invasive markers for NAFLD varies markedly according to the *PNPLA3* genotypes. Specifically, by evaluating the AUROCs of various non-invasive diagnostic markers with the CC and CG+GG variants of rs738409, we have observed important discrepancies in their diagnostic performance. Indeed, FLI and HSI's capabilities for accurately identifying steatosis from healthy controls were good only in the rs738409 CG+GG patients. Conversely, non-invasive diagnostic markers for NASH, namely CK18 M30 and M65, showed excellent diagnostic performance for predicting NASH only in the rs738409 CC patients, while non-invasive markers for fibrosis including BARD and FIB-4 showed good performance in differentiating significant fibrosis among NASH patients only in the rs738409 CG+GG and CC groups, respectively.

Some of the non-invasive diagnostic tests for NAFLD may have good utility in clinical practice. However, their diagnostic performance varies greatly between populations. For example, hepatocyte apoptosis and necrosis markers CK18 M30 and CK18 M65 showed promising utility for predicting NASH.[18] However, a meta-analysis of eleven studies for CK-18 fragments found a pooled AUROC of 0.71-0.93 with sensitivity and specificity for NASH of 66% and 82%, respectively.[19] Our evaluation of diagnostic performance of CK18 M30 and M65 also confirmed this discrepancy between different *PNPLA3* genetic groups. In another study, Borman *et al.* assessed the diagnostic performance of FLI in three subgroups.[20] In each of these 3 subgroups,

FLI showed an AUROC of 0.68, 0.68 and 0.60 for hepatic steatosis, respectively. These non-invasive markers share the disadvantage of limited accuracy when they are used alone, with a wide range of cut-offs, as well as a lack of consistency in validation studies. These data suggest that these non-invasive diagnostic markers are not still ready for routine clinical practice.[21] Our findings provide novel insight into the variable utility of these non-invasive diagnostic tests suggesting that their inconsistency and low accuracy might be, at least in part, attributable to differences in *PNPLA3* polymorphism.

The reasons for the observed inconsistent results of the non-invasive markers for diagnosing and staging NAFLD according to *PNPLA3* genotypes require further examination. Indeed, multiple studies have linked *PNPLA3* genotypic variation to differences in NAFLD severity,[22-28] and rs738409 polymorphism is strongly associated with liver damage.[24, 27] *PNPLA3* is located in endoplasmic reticulum and lipid droplet membranes of the hepatocytes, and is regulated by insulin levels mainly through sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP).[8, 29] Previous studies have suggested that the *PNPLA3* I148M variant reduces the lipolytic activity and accumulation of lipid droplets into the liver.[30-32] However, the molecular mechanisms underlying this process are still unclear. Three new findings may account for how *PNPLA3* I148M contributes to development of NAFLD: 1) increased binding of *PNPLA3* to comparative gene identification-58 (CGI-58) resulting in reduced availability of CGI-58 to co-activate adipose triglyceride lipase (ATGL); 2) resistance to degradation resulting in increased abundance of *PNPLA3* protein; and 3) downregulation of autophagy of *PNPLA3* resulting in reduced lipolysis.[33] *PNPLA3* is associated with decreased kidney function not only in adults but also in children and adolescents with

NAFLD.[34, 35] In these two studies, subjects with GG genotype had lower e-GFR and higher 24-hour proteinuria or albuminuria compared with those with either CG or CC genotypes. Another study suggested dissociation of sarcopenia and NAFLD in rs738409 GG carriers, compared to CC and CG groups.[36] In addition, *PNPLA3* may also play a role in alcoholic liver disease and other chronic liver diseases.[8]

Given the primary role of *PNPLA3* in the risk of progressive NAFLD, the patients with NAFLD, who are homozygous for this variant allele (*PNPLA3*-148MM), have a genetic form of NAFLD that may have different risks of developing hepatic and extra-hepatic complications, and that has been named as "*PNPLA3*-related NAFLD". NAFLD is traditionally considered as the hepatic manifestation of the metabolic syndrome. However, numerous studies have suggested that NAFLD due to *PNPLA3*-148MM is not associated with increased BMI, atherogenic dyslipidaemia, or insulin resistance.[37] Another study has also suggested that patients with *PNPLA3*-related NAFLD do not have adipose tissue inflammation in contrast to those with NAFLD associated with obesity/metabolic syndrome.[9] Although further research is certainly needed, preliminary evidence suggests that "*not all forms of NAFLD were created equal*" and that the phenotype of patients with *PNPLA3*-related NAFLD is often different from that of patients with metabolic syndrome-related NAFLD who do not have the *PNPLA3*-148M variant.[38]

 We hypothesize that the *PNPLA3* I148M variant could have fundamental influence over the consistency of non-invasive diagnostic tests for NAFLD. Thus, *PNPLA3* rs738409 genotyping could be a useful screening test in NAFLD. However, better specific prediction models are needed to improve diagnostic accuracy for the different stages of disease severity in NAFLD. By

identifying patients' SNP genotype, it may prove possible to improve diagnostic accuracy and decrease the overall economic burden associated with a misdiagnosis in the assessment of NAFLD (*e.g.*, as proposed in Figure 2).

Limitations & Conclusions

Our study is subject to some important limitations. This is a single-center, cross-sectional study that cannot predict the disease progression. Due to the relatively small sample size of the study, the proposed cut-offs of non-invasive markers for diagnosing and staging NAFLD might not be generalizable to other populations with NAFLD of different ethnicity. Further research is needed to validate the optimal cut-offs of these non-invasive markers in larger independent series. Finally, since SNP analysis is still reserved only for research purposes, the cost for SNP testing and the time involved might represent an important limiting factor in reducing their routine clinical implementation.

In conclusion, our results show that the diagnostic performance of non-invasive tests for NAFLD varies markedly according to *PNPLA3* genotypes. We consider that clinicians should be aware that the *PNPLA3*-I148M (rs738409) genotype modifies the clinical utility of non-invasive diagnostic tests for diagnosing liver disease severity in NAFLD.

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TABLES

Table 1. Baseline characteristics of healthy control individuals and patients with biopsy-proven NAFLD.

Table 2. Baseline characteristics of non-NASH and NASH patients.

Table 3. Baseline characteristics of non-significant and significant fibrosis in NASH patients.

Table 4. Diagnostic performances of non-invasive markers for diagnosing and staging NAFLD.

FIGURE LEGENDS

Figure 1. Flowchart for the study.

Figure 2. A proposed flowchart for the diagnosis of NAFLD in clinical practice using SNP

genotyping.