Title

PNPLA3 rs738409 C>G variant influences the association between visceral fat and significant fibrosis in biopsy-proven NAFLD

Running title

Visceral fat in NAFLD

Authors' names

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Abbreviations:

ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyltransferase; HDL: high-density lipoprotein; LDL: low-density lipoprotein; HOMA-IR: homeostatic model assessment for insulin resistance; NAFLD: nonalcoholic fatty liver disease; SF: significant fibrosis; VFA: visceral fat area; PNPLA3: patatin-like phospholipase domain-containing protein 3.

Conflict of interest disclosure

All authors: nothing to declare.

Author's contributions

Study concept and design: Gang Li and Ming-Hua Zheng

Acquisition of data: Gang Li, Hong-Lei Ma, Liang-Jie Tang, Ou-Yang Huang, Xiao-Yan Pan, Pei-Wu Zhu, Rafael S. Rios, Kenneth I. Zheng

Pathology analysis: Sui-Dan Chen

Drafting of the manuscript: Gang Li, and Ming-Hua Zheng

Critical revision: Giovanni Targher and Christopher D. Byrne

Statistical analysis: Gang Li

Study supervision: Ming-Hua Zheng

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

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The study was approved by the local ethics committee of our hospital.

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Abstract

Background: Intra-abdominal visceral fat accumulation and *PNPLA3* (patatin-like phospholipase domain containing 3) rs738409 G/C gene polymorphism confer a greater susceptibility to nonalcoholic fatty liver disease (NAFLD). We examined whether the relationship between visceral fat accumulation and liver disease severity may be influenced by *PNPLA3* rs738409 polymorphism.

Methods: The variant of *PNPLA3* rs738409 was genotyped within 523 Han individuals with biopsy-confirmed NAFLD. Visceral fat area (VFA) was measured by bioelectrical impedance. Significant liver fibrosis (SF), defined as stage F ≥2 on histology, was the outcome measure of interest.

Results: The distribution of *PNPLA3* genotypes was CC: 27.5%, CG: 48.2%, and GG: 24.3%. Higher VFA was associated with greater risk of having SF (adjusted-odds ratio (OR) 1.03; 95%CI 1.02-1.04, P<0.05), independent of potential confounders. Among subjects with the same VFA level, the risk of SF was greater among carriers of the rs738409 G genotype than among those who did not. Stratified analysis showed that *PNPLA3* rs738409 significantly influenced the association between VFA and SF. VFA remained significantly associated with SF only among the rs738409 G-allele carriers (adjusted-OR 1.05; 95%CI 1.03-1.08 for the GG group; and adjusted-OR 1.03; 95%CI 1.01-1.04 for the GC group). There was a significant interaction between VFA and *PNPLA3* rs738409 genotype ($P_{interaction}$=0.004).

Conclusion: *PNPLA3* rs738409 G allele has a moderate effect on the association between VFA and risk of having SF in adult individuals with biopsy-proven NAFLD.

The existence of the *PNPLA3* rs738409 G allele and VFA interact to increase risk of SF.

Keywords

Nonalcoholic fatty liver disease; significant fibrosis; visceral fat area; SNP.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a major health problem that affects up to nearly 30% the world's adults.[1-3] NAFLD refers to a spectrum of progressive liver conditions ranging from simple steatosis (NAFL) to steatohepatitis (NASH) with varying amounts of fibrosis, and cirrhosis.[4,5] Convincing evidence shows that increased intra-abdominal visceral fat accumulation is a strong predictor for the development of significant liver fibrosis (SF) in NAFLD.[6,7] Unlike subcutaneous adipose tissue, visceral adipose tissue is anatomically related to the liver through the portal vein, and so the liver is directly exposed to higher levels of free fatty acids as well as multiple adipokines/cytokines directly released from expanded visceral adipose tissue within the portal vein, thereby promoting the development of NAFLD. Therefore, visceral fat accumulation is a key target for therapeutic interventions of NAFLD and other metabolic disorders.[8]

It is known that NAFLD is a complex and heterogeneous disease.[9,10] Studies show ~20% of adults may have NAFLD, in the absence of overweight or obesity.[11] The patatin-like phospholipase domain containing-3 (*PNPLA3*) rs738409 C >G variant (wild type to mutant) is one of the strongest genetic variants that is related to a greater susceptibility to developing NASH and cirrhosis.[12-14] Previous studies show that individuals with NAFLD, who carry the *PNPLA3* rs738409 G allele, do not have insulin resistance or other features of metabolic syndrome.[13,15,16] Preliminary studies also suggest that the *PNPLA3* rs738409 GG genotype is associated with a lower risk

of type 2 diabetes and cardiovascular disease;[17] thereby supporting the notion that the pathophysiology of NAFLD may be different among subjects carrying this genetic variant.

Thus, considering the possible differences in the pathophysiology of metabolic-related vs. PNPLA3-related NAFLD,[18] we have tested whether *PNPLA3* rs738409 may influence the effect of VFA on risk of having SF, and whether there is interaction between visceral fat content and *PNPLA3* rs738409 polymorphisms, to affect liver disease severity, within a well-identified cohort of subjects with biopsy-confirmed NAFLD.

Methods

Research population

This is a cross-sectional analysis of our well-characterized Prospective Epidemic Research Specifically Of NASH (PERSONS) cohort of 1015 ethnic Han adults with suspected NAFLD (mainly based on abnormal serum liver enzyme levels and/or evidence of hepatic steatosis on imaging techniques), who were admitted to the First Affiliated Hospital of Wenzhou Medical University (China) from July 18, 2017 to December 4, 2019, and who accepted to undergo liver biopsy. As detailed in **Figure 1**, 492 individuals were excluded for the following main reasons: (1) hepatocyte steatosis ≤5% on histology (n=80); (2) excessive alcohol intake (>140 g/week for men and >70 g/week for women, respectively) (n=112); (3) other secondary causes for

hepatic steatosis (n=228); and (4) missing data for *PNPLA*-3 rs738409 genotype, or Bioimpedance using InBody 720 (n=72). As a consequence of these exclusion criteria, a total of 523 adult individuals with NAFLD were included the final analysis. The study protocol was approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University (protocol number #2016-246, 1 December 2016). All participants signed a written informed consent to participate in this study.

Laboratory and clinical data

Samples of venous blood were harvested after at fast of least 8-hour within all patients. Biochemical parameters were evaluated by employing an automated analyzer (Abbott AxSYM) centrally. Homeostasis model assessment of resistance of insulin (HOMA-IR) was calculated as follows: fasting insulin (mIU/l) × glucose (mmol/l) / 22.5. Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. Obesity/overweight was identified as BMI ≥25 kg/m^2. Diagnostic criteria for hypertension and diabetes have been described in our previous studies.[19] Visceral fat area(VFA) was measured within 1 day of liver biopsy. A bioelectrical impedance analyzer (BIA) (InBody 720; Biospace, land Seoul, Korea) was used to measure VFA.[20,21] The aforementioned laboratory and anthropometric variables were collected in all participants within 1 day of liver biopsy examinations.

Liver biopsy

Liver biopsy procedures have been described within detail previously.[22] Briefly,

NAFLD was defined as histological evidence of >5% of steatotic hepatocytes. Subjects with a NAFLD Activity Score (NAS) ≥5 (having a score of at least 1 for each histological component: hepatic steatosis, lobular inflammation and ballooning) were diagnosed as having definite NASH. Fibrosis stages were graded from 0 to 4, based on the Brunt's histological criteria.[23] Significant liver fibrosis (SF) was defined as having a histological stage F≥2.[24]

Analysis of *PNPLA3* rs738409 polymorphism

As described previously,[25] the MassARRAY platform (Agena Bioscience, San Diego, CA, USA) was used to assess genotype of *PNPLA3* rs738409. For this genotype, we used ~20ng of genomic DNA from peripheral blood leukocytes. Locus-specific polymerase chain reaction (PCR) as well as primers for detection were designed by Assay Design Suite v3.1. Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry was used for detection of allele type followed by amplification of DNA by multiplex PCR.

Statistical analysis

Continuous variables were expressed as means ± SD or medians with interquartile ranges (IQRs), based on whether the distribution was normal or skewed, and then compared using the unpaired Student's t-test or the Mann-Whitney test as appropriate. Categorical variables were expressed as proportions and compared using the chi-squared test or the Fisher's exact test as appropriate. The chi-square test was also used

to test whether *PNPLA3* rs738409 genotypes were in Hardy-Weinberg equilibrium. The association between VFA and presence of SF (defined as stage F ≥2 on liver histology) was tested by binary logistic regression analysis. In these regression models, the association was adjusted for known risk factors and potential confounders, such as sex, age, obesity/overweight, hypertension, type 2 diabetes, HOMA-IR score, serum total cholesterol, triglyceride and albumin levels. Stratified and interaction analyses were also performed to examine the effect of *PNPLA3* rs738409 polymorphism on the association between VFA and SF. All data were analyzed with statistical packages R (The R Foundation; http://www.r-project.org; version 3.4.3) and Empower (R) (www.empowerstats.com, X&Y solutions, inc. Boston, Massachusetts).

Results

Baseline characteristics

A total of 523 Chinese individuals with biopsy-confirmed NAFLD were enrolled in this study. Subjects had a mean age of 42 years and 73.8% were men. 102 (19.5%) subjects had SF (stage F ≥2 on liver histology). The prevalence of hypertension and type 2 diabetes was 24.1% and 25.8% respectively. The distribution of *PNPLA3* rs738409 genotypes was as follows: 144 (27.5 %) had CC genotype, 252 (48.2%) had GC genotype, and 127 (24.3%) had GG genotype, respectively. This genotype distribution did not deviate from Hardy-Weinberg equilibrium. The frequency of the *PNPLA3* rs738409 G variant was 0.48, similar to a previous study from China

(0.45).[26] **Table 1** summarizes the baseline characteristics of study participants, stratified by *PNPLA3* rs738409 polymorphism. Carriers of the *PNPLA3* GG genotype had a significantly higher prevalence of severe steatosis and definite NASH. The three groups were well comparable in terms of sex, age, adiposity measures (including VFA), HOMA-IR score and other metabolic parameters. Notably, as shown in **Table 2**, after stratifying by both *PNPLA3* rs738409 polymorphism and SF, values of VFA were significantly greater only among carriers of the G allele, who also had SF.

***PNPLA3* rs738409 polymorphism influences the association between VFA and SF**

The smoothing spline curve, obtained by a generalized additive model, shows a linear association between VFA and risk of having SF. As shown in **Figure 2**, as VFA increased, the likelihood of SF also progressively increased; however, it is worth noting that carriers of the *PNPLA3* CC genotype had a lower risk of SF than those carrying the *PNPLA3* G allele. Among individuals with the same level of VFA, the risk of SF was higher among carriers of the rs738409 G genotype than among those who did not. The smoothing spline curve clearly suggested that *PNPLA3* rs7387409 G allele increased the probability of SF with increasing levels of VFA. A threshold effect analysis was also performed to examine if the slight fall in the probability of SF in the CC group with increasing levels of VFA was statistically significant. Although there are just 11 subjects in the descending section of the curve (**Figure 2**), we found that the fall in the probability of SF in the CC group with increasing levels of VFA was not statistically significant.

Association between VFA and SF

As reported in **Figure 3**, within a logistic regression model with the presence or absence of SF as the dependent variable, there was a significant positive association between VFA (included as a continuous variable) and risk of having SF, even after adjustment for sex, age, obesity/overweight, hypertension, type 2 diabetes, HOMA-IR score, serum total cholesterol, triglyceride and albumin levels (adjusted-OR 1.03; 95% CI 1.02-1.04).

Association between VFA and SF in different subgroups

We examined the association between VFA and risk of having SF in individuals, who were stratified either by different *PNPLA3* genotypes (additive or dominant models) or by other established risk factors for SF (i.e., sex, age, BMI, hypertension, diabetes and HOMA-IR score). As shown in **Figure 3**, the significant association between VFA and SF persisted in the *PNPLA3* rs738409 GG and GC subgroups even after adjustment for potential confounders (adjusted-OR 1.03, 95%CI 1.01-1.04 in the GC group; and adjusted-OR 1.05, 95%CI 1.03-1.08 in the GG group), but not in the CC group (adjusted-OR 1.01, 95%CI 0.99-1.03). It should be noted, there was a significant interaction of *PNPLA3* rs738409 genotypes on the association between VFA and risk of SF ($P_{interaction}$=0.004). When this association was assessed in a dominant genetic model, the association between VFA and SF remained statistically significantly after controlling for potential confounding variables in the GC+GG

group (adjusted-OR 1.03, 95%CI 1.02-1.05), but not in the CC group (adjusted-OR 1.01, 95%CI 0.99-1.03). In addition, there was a significant association between VFA and SF when we stratified subjects into two groups, i.e. the GG vs. GC+CC subgroups. Interestingly, there was an interaction between PNPLA3 rs738409 GG and VFA ($P_{interaction}$=0.004). These results suggest that the *PNPLA3* rs738409 G allele and VFA interacted to moderately increase the risk of having SF.

Stratified analyses according to sex

We examined the association between VFA with SF, stratified by a *PNPLA3* rs738409 dominant model, both in men and in women separately. As described in **Table 3**, in the unadjusted models, VFA was associated with an increased risk of having SF in both sexes. After adjustment for age, overweight/obesity, type 2 diabetes and hypertension, levels of serum total cholesterol, triglycerides, albumin, and HOMA-IR score (adjusted models 2), the association between VFA and SF remained statistically significant in both men and women (adjusted-OR 1.03, 95% CI 1.01-1.04 for men, and adjusted-OR 1.02, 95% CI 1.0-1.05 for women). However, as also shown in **Table 3**, after further stratification by *PNPLA3* rs738409 genotypes (CC vs. GC+GG groups), the significant association between VFA and SF disappeared among carriers of the rs738409 CC genotype (adjusted-OR 1.01 95% CI 0.99-1.04 for men; and adjusted-OR 0.98, 95% CI 0.95-1.02 for women, respectively). In contrast, the association between VFA and SF remained significant among carriers of the rs738409 G allele, even after adjustment for potential confounders in both sexes (adjusted-OR

1.03, 95%CI 1.02-1.05 for men; and adjusted-OR 1.04, 95%CI 1.01-1.07 for women, respectively).

Discussion

In this large cross-sectional study of ethnic Han individuals with biopsy-confirmed NAFLD, we found that intra-abdominal VFA was significantly associated with greater risk of having SF, on liver histology. Notably, this significant association persisted even after adjusting for potential confounding variables, such as sex, age, obesity/overweight, hypertension, diabetes, HOMA-IR score, plasma lipids and albumin levels. Furthermore, after further stratification by *PNPLA3* rs738409 polymorphism, the association between VFA and SF remained significant only among carriers of the *PNPLA3* rs738409 G allele, but not among those carrying the CC genotype, thereby suggesting that the *PNPLA3* rs738409 G allele and VFA can interact to moderately increase the risk of having SF. Furthermore, with the same level of VFA, the risk of having SF was significantly lower among carriers of the rs738409 CC genotype than among those carrying the rs738409 G allele.

In the last decade, the close inter-relationship between intra-abdominal VFA and SF in people with NAFLD has drawn increasing attention.[6,7] Unlike subcutaneous fat in the abdomen, intra-abdominal visceral fat accumulation (being connected directly to the liver via the portal vein) is closely related to the development and progression of NAFLD.[8,27] In our study, we found that VFA was associated with greater risk of SF,

independently of pre-existing diabetes or other metabolic syndrome features, especially among carriers of the *PNPLA3* CG or GG genotypes. The precise mechanisms underpinning the association between increased VFA and greater risk of SF are not fully understood. However, in accord with the so-called "portal theory", it has been proposed that expanded and dysfunctional visceral adipose tissue may release higher amounts of free fatty acids as well as multiple adipokines and pro-inflammatory cytokines into the liver via portal vein, thus promoting the development and progressions of NAFLD.[28-31]

We found that compared to NAFLD subjects carrying the *PNPLA3* rs738409 CC genotype, VFA was independently related to a greater risk of having SF only among those carrying the *PNPLA3* rs738409 G-allele. It is known that the *PNPLA3* rs738409 C>G variant (wild type to mutant), leading to an isoleucine to methionine substitution at position 148 of the protein (I148M), is strongly associated with an increased risk of NAFLD progression. As a liver lipase with triglyceride hydrolase enzyme activity, this genetic variant leads to loss of function, thereby reducing the remodeling of polyunsaturated fatty acids and monounsaturated fatty acids, leading to their retention within the liver.[32] Therefore, it is conceivable that the combination of increased VFA and *PNPLA3* rs738409 C>G variant may promote the progression of NAFLD from simple steatosis to NASH and cirrhosis.

We believe that the presence of an interaction effect of *PNPLA3* rs738409 G allele

and VFA to moderately increase risk of SF, and the observed dissociation of VFA and SF among the carriers of the *PNPLA3* rs738409 CC genotype are two interesting findings of our study. However, the specific reasons for these results are not entirely known. In particular, the effect as well as role of the *PNPLA3* rs738409 G variant within adipose tissue are poorly understood. Recently, it has been shown that PNPLA3 mRNA was expressed abundantly within the liver and detectable clearly also within the subcutaneous adipose tissue of individuals with severe obesity.[33] Other investigators confirmed that PNPLA3 protein was found not just within the liver, but also within adipose tissue. It has been reported that *PNPLA3* rs738409 C>G variant may alter lipid composition of adipose tissue in a similar way to that observed in the liver.[34,35] An experimental study also suggested that overexpression of the PNPLA3 rs738409 G variant lead to greater VFA and insulin resistance compared to the wild type protein in mice.[36] Although it remains still uncertain how the *PNPLA3* rs738409 polymorphism may interact with VFA to increase hepatic fibrogenesis, our results support the existence of a cross-talk between VFA and *PNPLA3* rs738409 polymorphism on risk of NAFLD progression.[34]

In our study we enrolled patients with biopsy proven NAFLD who also had measurement of visceral fat area and PNPLA3 SNP status. There was a significant trend for patients with more significant fibrosis (stage 2 or more) to have increased VFA or be a carrier for the G allele. Consequently, we thought it would also be a valuable point if those with negative biopsies were analyzed to understand if the VFA

was significantly different in this population as well as the status of PNPLA3. Unfortunately, we didn't enroll this part of patients in our cohort. And further studies are required for this point in the future.

There are several essential limitations within our research. Firstly, the mutation rates of PNPLA3 rs738409 polymorphism vary among different ethnic populations, with highest rates in Asian and American individuals, intermediate rates in northern European whites, and lowest rates in blacks. For example, according to a previous study the risk allele mutation for PNPLA3 was 49% among Hispanics, followed by non-Hispanic Caucasians (23%) and African Americans (17%) [16,37]. As the participants in our study were all ethnic Han Chinese individuals, the findings of our study might not generalizable to other ethnic groups.[38-40] Secondly, the cross-sectional design of our study does not allow any firm conclusions about causality. However, since *PNPLA3* rs738409 polymorphism is inherited, reverse causation does not apply. Thirdly, VFA was not measured with computed tomography (CT) scan. However, VFA estimated by BIA has a good correlation with VFA measured with CT scaning.[41] Finally, we did not have detailed information on physical activity levels and diet regimens of these participants. It is well known the beneficial effect of different exercise regimes, without caloric restriction, on VFA in overweight or obese individuals.[42]

In conclusion, our research showed that VFA is associated with greater risk of having

SF, independently of potential confounding factors, especially among carriers of the *PNPLA3* rs738409 G-allele, and there is an interaction of *PNPLA3* rs738409 polymorphism and VFA to increase risk of SF in Chinese individuals with biopsy-proven NAFLD. Our gene-visceral fat interaction study suggests that the *PNPLA3* rs738409 G-allele may moderately modulate the adverse effects of VFA on risk of SF in NAFLD. However, further research is needed to further corroborate these findings in other different cohorts of NAFLD patients.

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Table Legends

Table 1. Baseline characteristics of study participants, stratified by *PNPLA3* rs738409 polymorphism.

Table 2. Baseline characteristics of study participants, stratified by both *PNPLA3* rs738409 polymorphism and significant liver fibrosis (SF).

Table 3. Associations between visceral fat area and significant liver fibrosis in participants with different *PNPLA3* genotypes, stratified by sex.

Figure Legends

Fig 1. The flowchart of the study.

Fig 2. Association between visceral fat area and significant liver fibrosis (SF) in biopsy-proven NAFLD, stratified by *PNPLA3* rs738409 polymorphism.

Fig 3. Associations between visceral fat area and significant liver fibrosis in different subgroups of individuals. All data are adjusted for age, sex, type 2 diabetes, hypertension, body mass index, serum total cholesterol, triglycerides, albumin levels and HOMA-IR score (with the exception of the specific variable used for stratifying each patient subgroup).