***Title*: Association between positivity of serum autoantibodies and liver disease severity in patients with biopsy-proven NAFLD**

**Short Title:** Serum autoantibodies in NAFLD

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**List of Abbreviations**

NAFLD: nonalcoholic fatty liver disease; AIH: autoimmune hepatitis; NASH-CRN: nonalcoholic steatohepatitis clinical research network; ANA: anti-nuclear antibodies; PNPLA3: patatin like phospholipase domain containing 3; PBC: primary biliary cholangitis; anti-CENP-B: anti-centromere antibody; AHA: anti-histone antibody; AMA: anti-mitochondrial antibody; SLA: soluble liver antigen; ASMA: anti-smooth muscle antibody; LKM: liver-kidney microsomal antibody; LC-1: liver cytosol antibody type 1; ELISA: enzyme-linked immunosorbent assay; HOMA-IR: homoeostasis model assessment of insulin resistance; SNP: single nucleoid polymorphism; MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization-Time of Flight; IFN: interferon; TNF: tumor necrosis factor; IL: interleukin.

**Ethics approval statement:** Ethical approval for the study was obtained from the ethics committee of the First Affiliated Hospital of Wenzhou Medical University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the ethical guidelines of the 1975 Declaration of Helsinki.

**Informed consent:** Informed consent was obtained from all individual participants included in the study.

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**ABSTRACT**

**Background and Aims:** Some previous studies reported serum autoantibody positivity in patients with nonalcoholic fatty liver disease (NAFLD). The clinical significance of these findings remains uncertain. We aimed to investigate the association between the presence of serum autoantibodies and liver disease severity in NAFLD.

**Methods:** A total of 388 consecutive patients with biopsy-proven NAFLD were included in the study. Various serum autoantibodies (including also anti-nuclear antibodies [ANA]) were detected by indirect immunofluorescent or immunoblotting assays.

**Results:** Overall, 84 (21.6%) patients with biopsy-confirmed NAFLD had positivity for at least one of the measured serum autoantibodies. ANA positivity was present in 50 (12.9%) patients, whereas anti-U1RNP or pANCA antibodies were detectable in 9 (2.3%) and 6 (1.5%) patients, respectively. Multivariate logistic regression analysis showed that ANA positivity (adjusted-odds ratio: 4.51, 95%CI: 1.77-11.5; P=0.002) or positivity of any serum autoantibodies (adjusted-odds ratio: 3.14, 95%CI: 1.30-7.62; P=0.01) were independently associated with advanced liver fibrosis (stages F3-F4). In serum autoantibody/ANA-positive patients, the proportion of those with advanced fibrosis was also greater among carriers of *PNPLA3* rs738409 GG or CG than among those carrying *PNPLA3* rs738409 CC genotype.

**Conclusions:** Serum autoantibody positivity was independently associated with advanced liver fibrosis in patients with biopsy-proven NAFLD. The presence of serum autoantibodies in patients with advanced fibrosis occurred more frequently amongst those carrying *PNPLA3* rs738409 GG or CG genotypes.

**Keywords:** Autoantibodies, Fibrosis, NAFLD, Liver biopsy

**BACKGROUND**

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver diseases worldwide, and poses a health threat in nearly a quarter of the Asian adult population [1]. Serum autoantibodies refer to the immunoglobulins reactive to autoantigens inside, on the surface, or outside the cell, which reflect autoimmunity and may serve as biomarkers for many autoimmune disorders. Although the pathogenesis of NAFLD is strongly associated with overweight/obesity, insulin resistance and other metabolic risk factors rather than autoimmunity [2, 3], the presence of one or more serum autoantibodies may occur in ~20-35% of patients with NAFLD, who do not have coexisting autoimmune hepatitis (AIH) [4-6].

Previous reports examining the clinical significance of serum autoantibodies in individuals with NAFLD have yielded inconsistent results. For example, a study of 225 United States patients with biopsy-proven NAFLD has shown that serum autoantibody positivity was associated with greater histological severity of NAFLD (especially liver fibrosis and necro-inflammation) [4]. In contrast, in the nonalcoholic steatohepatitis clinical research network (NASH-CRN) database, Vuppalanchi et al. did not find any association between the presence of serum autoantibodies and more advanced histologic features of NAFLD in a cohort of 864 patients with biopsy-proven NAFLD [7]. Additionally, Ravi et al. have shown that serum autoantibody positivity was not associated with clinical presentation and clinical outcomes in patients with NAFLD or alcohol-related liver disease in the USA [6]. Another small study of 84 Italian patients with NAFLD found that anti-nuclear antibodies (ANA) positivity (>1:100 titers) was associated with markers of insulin resistance [5].

To our knowledge, in Asian populations, there is only one published study performed in 212 Japanese patients with NAFLD, that did not find any association between severity of NAFLD histology and serum positivity for ANA [8]. To date, studies examining the association between autoantibody positivity and NAFLD severity in the Chinese population are lacking. Since heterogeneity may exist between different ethnic groups, it is important to investigate whether there are associations between positivity of serum autoantibodies and liver disease severity across different ethnic groups.

It is well known that the patatin like phospholipase domain containing 3 (*PNPLA3*) I148M genotype (rs738409) is the most robust single genetic variant influencing disease severity and progression of NAFLD [9, 10], but it is currently not known whether this genetic variant is also associated with serum autoantibody positivity in NAFLD.

Since it remains uncertain whether the presence of serum autoantibodies is associated with liver disease severity in patients with NAFLD, the main aim of our study was to investigate the association between serum autoantibodies and liver disease severity in a cohort of Chinese patients with histologically-proven NAFLD.

**MATERIALS AND METHODS**

***Study population***

We consecutively recruited adult individuals with biopsy-proven NAFLD at the First Affiliated Hospital of Wenzhou Medical University between December 2016 and January 2019. Detailed inclusion and exclusion criteria have been described previously [11, 12]. Briefly, from an initial cohort of 739 patients with suspected NAFLD (based on the imaging evidence of hepatic steatosis and/or elevated serum liver enzymes), we ruled out cases with other known causes of liver disease, such as alcohol-related liver disease, drug-induced hepatitis, viral hepatitis, autoimmune hepatitis (AIH), Wilson’s disease and primary biliary cholangitis (PBC). Patients with history of significant alcohol consumption (>20 g/day in women and >30 g/day in men) were excluded by a questionnaire-based lifestyle survey. We also excluded some patients with serum anti-Ro52 or anti-M2-3E positivity, who probably deserve more in-depth assessment of other coexisting autoimmune/rheumatic diseases.

As a consequence, a total of 388 patients with biopsy-proven NAFLD, who did not report any prior history of autoimmune or rheumatic diseases, were included in the final analysis. Liver histology data confirmed there were no pathologic features of AIH (interface hepatitis, plasma cell infiltration, hepatic rosette formation, or emperipolesis) [13] or PBC (chronic non-suppurative destructive cholangitis, ‘florid duct lesions’) [14] in any of these patients. In particular, the AIH score proposed by the international autoimmune hepatitis group scoring system [12] was less than 6 in all patients enrolled in this study.

Ethical approval was obtained from the First Affiliated Hospital of Wenzhou Medical University Ethics Committee (2016-246, approved on 1 December 2016). The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. Each subject has provided written informed consent.

***Clinical parameters and autoimmune markers measurement***

For each patient, fasting blood samples were obtained on the same day of the liver biopsy examination. Serum autoantibodies, including ANA, anti-mitochondrial antibody (AMA), anti-dsDNA, anti-smooth muscle antibody (ASMA), and perinuclear anti-neutrophil cytoplasmic antibody (pANCA), were determined by indirect immunofluorescent assay. The substrate for ANA was human epithelial-2 (HEp-2) cells, the substrate for ASMA testing was rat stomach tissue, and the substrate for pANCA was neutrophilic granulocytes. Positive immunofluorescent tests were defined as presence of specific yellowish green fluorescence in cells or tissues. ANA with a titer ≥1:100 was considered positive, as suggested by Qiu *et al.* and the Chinese consensus on the diagnosis and management of AIH [15, 16]. Anti-U1RNP, anti-Sm antibody, anti-soluble liver antigen (anti-SLA), anti-Ro52, anti-nucleosome antibody, anti-liver-kidney microsomal antibody (anti-LKM), anti-liver cytosol antibody type 1 antibody (anti-LC-1), anti-PML protein antibody, anti-SSA, anti-SSB, anti-Scl-70, anti-Jo-1, anti-Rib-P, anti-centromere antibody (anti-CENP-B), anti-histone antibody (AHA), AMA-M2, anti-M2-3E, anti-gp210 antibody, and anti-sp100 antibody were measured by commercially available immunoblotting assay kits. The substrate for immunoblot tests was nitro-Blue-Tetrazolium/ 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP), and positive immunoblot tests were defined as detection of a clearly visible mark corresponding to the specific position on the blot. The positivity of autoimmune markers was defined as the presence of *at least* one of the aforementioned serum autoantibodies.

Other routine blood biochemistry assessments were performed by standard laboratory methods, for which a detailed description can be found in our previous studies [12]. Anthropometric measurements, including body mass index (BMI, weight/height2), waist circumference and hip circumference were measured in all patients. Hypertension was diagnosed based on blood pressure ≥ 140/90 mmHg or use of any antihypertensive drugs. Homoeostasis model assessment (HOMA-IR)-estimated insulin resistance was calculated in all non-diabetic patients with NAFLD as follows: fasting insulin (μU/mL) \* fasting glucose (mmol/L)/22.5. A diagnosis of type 2 diabetes mellitus was based on medical history, fasting glucose levels ≥ 7.0 mmol/L and/or glycated hemoglobin ≥ 6.5% [17].

***Histological evaluation of NAFLD***

Ultrasound-guided percutaneous liver biopsy was performed using 16G Hepafix needle. Formalin-fixed paraffin-embedded biopsy tissue with hematoxylin-eosin and Masson’s trichrome staining were interpreted by an experienced hepato-pathologist (Xiao-Dong Wang), who was blinded to patients’ clinical and laboratory data. Histological scoring of NAFLD was based on the NASH-CRN scoring system [18]. We defined NASH as presence of steatosis, ballooning and lobular inflammation on histology (at least one point for each) and NAFLD activity score (NAS) ≥4 [18]. The histological staging of liver fibrosis was determined according to the Brunt's criteria [19]. Presence of stages F3-F4 on histology was defined as advanced liver fibrosis, a condition which increases risk of hepatocellular carcinoma and is associated with poorer prognosis in NAFLD [20].

***Genetic analysis***

We extracted 20 ng genomic DNA from peripheral blood samples for single nucleoid polymorphisms (SNP) genotyping of *PNPLA3* rs738409. Genotyping assay for SNP loci were designed via the MassARRAY System (Agena Bioscience, San Diego, CA). After amplification of DNA samples through locus-specific polymerase chain reaction (PCR) according to Assay Design Suite software (Version 3.1), *PNPLA3* rs738409 allele detections were performed by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry.

***Statistical analysis***

Continuous variables were presented as mean ± standard deviation or median [interquartile range], and categorical data were presented as number (%). Continuous data with or without normal distribution were compared using the unpaired Student’s *t*-test or the two-sample Mann-Whitney U test, as appropriate. Categorical variables were compared using the chi-squared test or the Fisher’s exact test, as appropriate. The Cochran-Armitage trend test (chi-squared test for trend) was employed to examine the presence of association between serum autoantibody positivity and histological scoring of NAFLD via the “*CATT* ” R package, which has better accuracy than the Pearson’s chi-squared test for the assessment of associations between a variable with two categories and an ordinal variable with ≥ 3 categories [21, 22]. Statistical analyses were conducted using IBM SPSS version 22.0 and R version 3.6.0 (https://www.r-project.org/). For all statistical tests, a two-sided *P* value ≤ 0.05 was considered statistically significant.

**RESULTS**

***Patient characteristics***

A total of 739 consecutive patients with biopsy-proven fatty liver disease of any aetiology were initially enrolled. We excluded patients with alcoholic fatty liver (n = 105), those with probable or definite AIH (n = 8; according to the international AIH group scoring system), drug-induced hepatitis (n = 31), hepatitis virus-infected patients (n = 181), and those (n = 26) with either serum anti-Ro52 or anti-M2-3E positivity, who probably deserve more in-depth assessment for autoimmune/rheumatic diseases. Thus, a total of 388 patients with biopsy-confirmed NAFLD, who did not have any prior history of autoimmune or rheumatic diseases, were included in the final analysis.

**Table 1** shows the clinical, biochemical and liver histological features in patients with biopsy-proven NAFLD, stratified by positivity of any serum autoimmune antibodies.

The mean age of the study population was 40.6 years, and men accounted for 74.7% of the patients. We compared anthropometric measurements, laboratory parameters, SNP genotyping, and liver histological findings between patients, with and without serum autoantibody positivity. Among routine clinical and biochemistry biomarkers, only waist circumference showed a borderline significant difference between the two patient groups (*P* = 0.05). Interestingly, although no significant differences were found in prevalence of NASH (*P* = 0.20), histologic NAS score (*P* = 0.72) and its individual histologic components (steatosis, ballooning, lobular inflammation), a greater histologic severity of liver fibrosis was observed in serum autoantibody-positive patients than in those who were negative for all serum autoantibodies (*P* = 0.01). *PNPLA3* rs738409 genotypes were not significantly different between the two patient groups (*P* = 0.89). Additionally, we did not find any significant difference in thyroid function parameters between the two groups (**Table 1**).

***Autoimmune markers positivity landscape of NAFLD patients***

An UpSet plot summarizes the proportions and intersections of various serum autoantibodies found positive amongst patients with biopsy-proven NAFLD (**Figure 1**). A total of 84 (21.6%) patients had positivity for at least one of serum autoantibodies. ANA was positive in 50 (12.9%) patients, whereas anti-U1RNP and pANCA antibodies were present in 9 (2.3%) and 6 (1.5%) patients, respectively; anti-Jo-1 was present in 5 (1.3%) patients, and ASMA in 5 (1.3%) patients. Anti-sp100 was positive in 4 patients; AHA, and anti-LC-1 were found positive in 3 patients, respectively; anti-SSB, anti-Rib-P, AMA-M2, anti-SLA, and anti-PML were positive in 2 patients, respectively. Anti-SSA, anti-Scl-70, and anti-CENP-B was positive in 1 patient, respectively. None of these patients had positivity for anti-sp100, anti-LKM, AMA, anti-Sm antibody, anti-dsDNA or anti-nucleosome antibodies. Most patients with ANA positivity showed low titers (<1:100), except for two patients with ANA titers of 1:320 and one patient with 1:1000. Notably, immunofluorescent pattern of ANA was available only in 37 ANA-positive patients (74% of total), with ‘speckled’ pattern in 24 (64.9%) of these patients, ‘nucleolar’ pattern in 12 (32.4%) patients, and ‘peripheral’ pattern only in one patient, respectively.

***Association between ANA positivity and severity of liver fibrosis***

As reported in **Table 1**, we observed a significantly different distribution of liver fibrosis stages between serum autoantibody positive and negative patients. As shown in **Table 2**, an increased proportion of advanced fibrosis was found in patients with serum autoantibody positivity (14.3% vs. 5.3%, *P* = 0.005). More specifically, when we analyzed the association between advanced fibrosis and ANA positivity (i.e., the most frequently observed autoantibody in our NAFLD patients), we identified a nearly four-fold higher prevalence of advanced fibrosis in ANA-positive patients than in ANA-negative patients (20.0% vs. 5.3%, *P* < 0.001); we also observed a more than three-fold higher prevalence of advanced fibrosis in patients with both ANA and ASMA positivity (18.9% vs. 5.4%, *P* < 0.001; **Figure 2**).

Because NAFLD and autoimmune diseases have a definite sexual dimorphism [23, 24], we performed a subgroup analysis by sex in **Supplementary Table 1**. We found a significant association between ANA/ASMA positivity and advanced liver fibrosis in men (*P* < 0.05); however, a marginally significant *P* value (*P* = 0.09) was also observed in women.To test the independence of the association between serum autoantibody positivity and advanced fibrosis, we further undertook multivariable logistic regression analyses with advanced fibrosis (F3-F4 stages) as the outcome variable. **Table 3** shows the association between serum autoimmune antibodies (ANA positivity or any serum autoantibody positivity) and advanced liver fibrosis after adjusting for known risk factors and potentially confounding factors. We included sex as a categorical variable in multivariable regression models 1 and 2, and presence of women aged more than 50 years (as a surrogate index for menopausal status) in multivariable regression models 3 and 4. In these logistic regression models, sex was not independently associated with advanced liver fibrosis (adjusted-OR: 0.50; 95% CI 0.18-1.38, *P* = 0.19).

In regression model 1, the presence of ANA positivity was independently associated advanced fibrosis (adjusted OR 4.51; 95% CI 1.77-11.5, *P* = 0.002). Similarly, in regression model 2, the presence of any positivity of serum autoantibodies was also independently associated with advanced fibrosis (adjusted OR 3.14; 95% CI 1.30-7.62, *P* = 0.01). In regression models 3 and 4, we found that postmenopausal status (> 50 years old) was independently associated with an approximately 8-fold increased risk of having advanced fibrosis (*P* < 0.001), similar to previous reports [25]. However, even after adjusting for menopausal status, ANA positivity remained significantly associated with advanced fibrosis (adjusted OR: 6.42; 95% CI 2.39-17.2, *P* < 0.001).

***Role of PNPLA3 rs738409 polymorphism in the association between autoimmune markers and liver fibrosis severity***

*PNPLA3* rs738409 genotype was assessed in 367 NAFLD patients (94.6% of total). The frequencies of *PNPLA3* rs738409 CC and CG+GG genotype were 101 (27.5%) and 266 (72.5%), respectively. The proportion of patients with advanced fibrosis did not differ with serum autoantibody-positivity, ANA-positivity, or ANA&ASMA-positivity, amongst NAFLD patients carrying the *PNPLA3* CC genotype (*P* = 0.45, 0.38, and 0.46, respectively). In contrast, advanced fibrosis was more frequent in those carrying the *PNPLA3* GG or CG genotype (*P* = 0.001 and < 0.001, respectively; **Table 4**). We further analyzed the possible association between the positivity of serum autoantibodies and the *PNPLA3* SNP genotype, and found that there was no significant association between ANA positivity*,* ANA/ASMA positivity, or positivity of any serum autoantibodies with *PNPLA3* rs738409 polymorphism (all *P* values> 0.1; **Supplementary Table 2**).

**DISCUSSION**

This is the first large cross-sectional study to investigate the association between serum autoantibody positivity and histological severity of NAFLD that also includes *PNPLA3* rs738409 genotype in a large Chinese cohort of patients with biopsy-proven NAFLD. We found that 21.6% of our patients had positivity for at least one of the measured serum autoantibodies. We also found a significant association between positivity of serum autoantibodies and risk of having advanced liver fibrosis (stages F3-F4). Notably, the presence of serum autoantibody positivity in patients with advanced fibrosis occurred more frequently in those carrying the *PNPLA3* rs738409 GG or CG genotypes.

Interestingly, the overall prevalence of any serum autoantibody positivity was 26.6%, whereas the prevalence of ANA positivity was 14% in our cohort of patients. This finding is similar to previous reports in NAFLD [4]. Similar proportions of serum autoantibody positivity or ANA positivity have been reported in general adult populations [26-28], indicating that low titers of these serum autoantibodies are not uncommon in apparently healthy individuals. ANA-positive healthy individuals have been shown to have higher concentrations of serum pro-inflammatory cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β [28], and these subjects have a higher risk of developing connective tissue diseases [29]. In our study, 76.3% of autoantibody-negative NAFLD patients and 68.2% of autoantibody-positive NAFLD patients were men, which implies that prevalence of serum autoantibody positivity was (as expected) higher in women [27].

Our findings, obtained in Chinese patients with NAFLD of Han ethnicity, are consistent with those previously reported by Adams et al. [4], but not with findings reported by other investigators in the United States [6, 7]. Both our and Adams et al.’s results showed that the presence of serum autoantibodies was strongly associated with more advanced stages of fibrosis in NAFLD. However, Adams et al. also noted that serum autoantibody positivity was associated with increased necro-inflammatory grades, whereas we failed to observe this association.

The possible biological mechanism(s) linking serum autoantibody positivity and advanced fibrosis in NAFLD are not entirely understood. A plausible mechanism explaining this association could involve the increased production of multiple pro-inflammatory cytokines (directly stimulated by autoimmune antibodies), such as tumor necrosis factor-α and interleukin-1β, contributing to the development of liver fibrosis by activating hepatic stellate cells [30]. The results of our study also suggest a role for the *PNPLA3* rs738409 polymorphism in NAFLD patients with serum autoantibody positivity. It is known that *PNPLA3* I148M polymorphism is associated with greater susceptibility to liver disease severity in NAFLD [9, 10, 31, 32] and, notably, we found that the association between serum autoantibody positivity and advanced liver fibrosis was restricted to patients carrying the *PNPLA3* CG or GG genotypes. We speculate that this finding might partly be explained by the greater pro-inflammatory and pro-fibrotic effects of the additional burden induced by this NAFLD-related genetic variant.

Some important limitations of our study merit mention. First, the cross-sectional design of this single-center study does not allow to establish casual and temporal relationships between serum autoantibody positivity and advanced liver fibrosis. Second, our findings need further validation in other ethnic groups of well-characterized patients with NAFLD.

**Conclusion**

Our cross-sectional study shows for the first time that positivity of serum autoantibodies (present in nearly 22% of these patients) was independently associated with advanced liver fibrosis in a large cohort of Chinese patients with biopsy-confirmed NAFLD. The positivity of serum autoantibodies in patients with advanced fibrosis occurred more frequently amongst those carrying *PNPLA3* rs738409 GG or CG genotypes.

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**FIGURE LEGENDS**

**Figure 1.** UpSet plot of interactions between different serum autoantibody positivity in patients with biopsy-proven NAFLD.

**Figure 2.** Distribution of liver fibrosis stages in patients with NAFLD with/without serum autoantibody positivity. (A) stratified by positivity of any serum autoantibodies; (B) stratified by positivity of ANA; (C) stratified by positivity of both ANA and ASMA.